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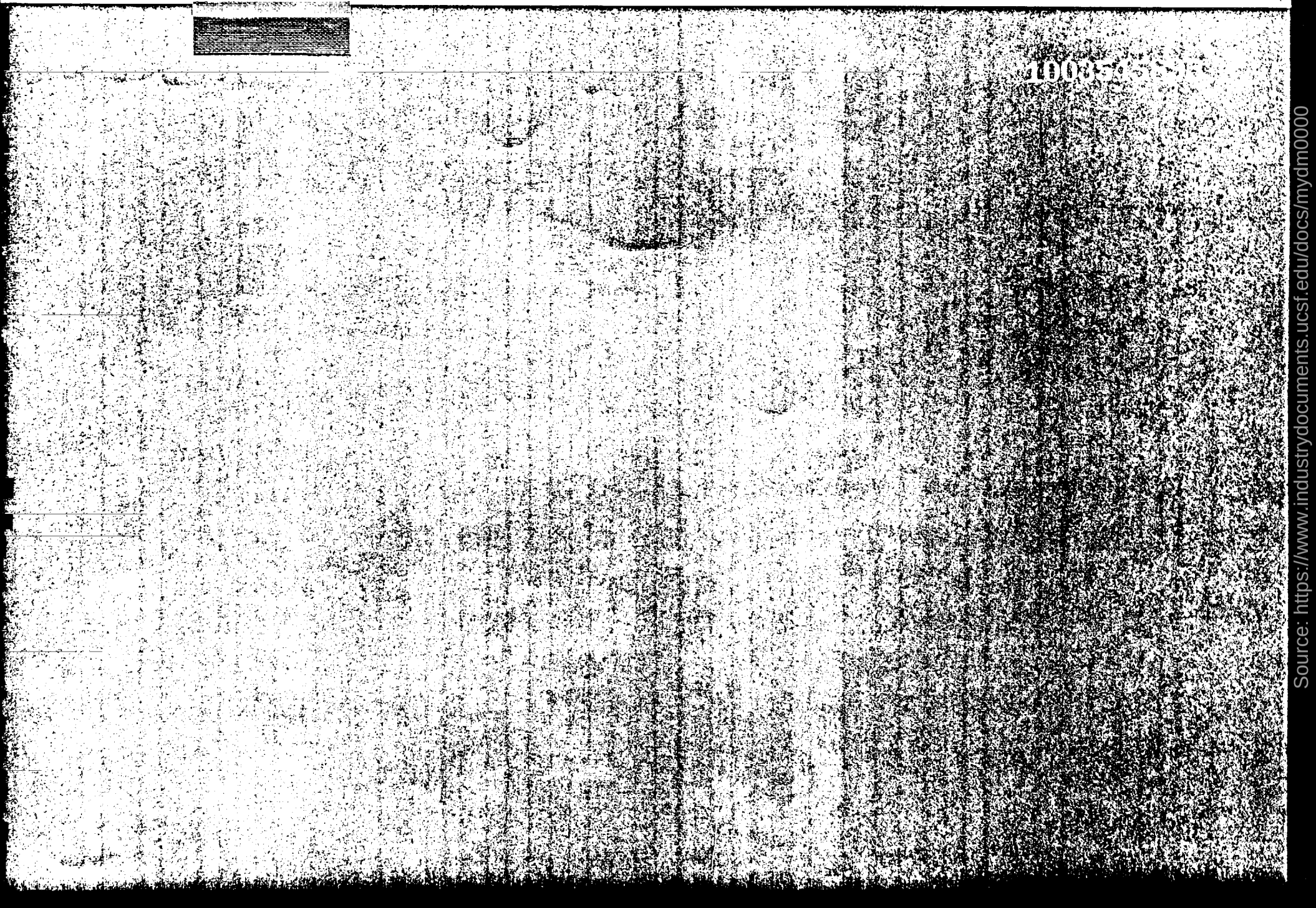
BOOK II

(94)

MEETING

SCIENTIFIC ADVISORY BOARD

September 10, 11, 12, 1975



#1048-BEDROSSIAN

[redacted]

1003545894

THE COUNCIL FOR TOBACCO RESEARCH – U.S.A., INC.

July 14, 1975

Grant application No. 1048

PULMONARY

To: The committee comprising Drs. Gardner, Lynch and Sommers

Subject: Carlos W. M. Bedrossian, M.D., University of Texas Medical School, Houston
New application No. 1048
"Relationship between Pulmonary Emphysema and Liver Cirrhosis on the Basis of an Acquired Deficiency of Alpha-1-Antitrypsin"

History

An informal inquiry was handled as Case No. 295, and the Executive Committee voted to encourage application (August 1974). The application was not submitted to CTR until June 1975. Dr. Bedrossian will be moving from the University of Oklahoma to the University of Texas in August 1975.

Request

Application No. 1048 requests $20,665 for the first year of a two year project. Estimate for the second year is $17,250.

Documents submitted (attached)

1. Application dated June 23, 1975 (6 pages).
2. C.V.s of Drs. Bedrossian, Cannon and Miller
3. First pages from twelve reprints (complete reprints will be submitted on request).

D.S.

DS:wg
Atts.

1003545895

#1048

The Council For Tobacco Research–U.S.A., Inc.

110 East 59th Street
New York, N. Y. 10022
(212) 421-8885

Application for Research Grant
(Use extra pages as needed)

Date: June 23, 1975

1. Principal Investigator (give title and degrees):
Carlos W. M. Bedrossian, M.D.
Assistant Professor of Pathology, University of Texas Medical School at Houston, Assistant Pathologist and Director of Cytopathology, Hermann Hospital, Houston, Texas.

2. Institution & address:
The University of Texas Health Science Center at Houston (Medical School)
P. O. Box 20036
Houston, Texas 77025

3. Department(s) where research will be done or collaboration provided:
Pathology Department

4. Short title of study:
Relationship Between Pulmonary Emphysema and Liver Cirrhosis on the Basis of an Acquired Deficiency of Alpha-1-Antitrypsin

5. Proposed starting date: January 1, 1976

6. Estimated time to complete: Two years

7. Brief description of specific research aims:
The purpose of this investigation is to determine the incidence and pattern of the emphysema present in association with advanced cirrhosis of the liver in an autopsy population of smoking and non-smoking alcoholics. This approach will be a continuation of our interest in studying the background factors and determining the relationship of emphysema to other conditions as we have previously done with heart disease[1], systemic hypertension[2] and bronchogenic carcinoma[3].

8. Brief statement of working hypothesis:

The reason to investigate the relationship between emphysema and cirrhosis is based on the hypothesis that the latter will result in an acquired deficiency of alpha-1-antitrypsin (A_1AT). As it is known, the liver is the site of production of A_1AT believed to be the principal inhibitor of the proteases[4]. Congenital deficiency of this enzyme was first associated with emphysema appearing in young persons[5]. Subsequently, lack of A_1AT has been linked to the development of cirrhosis in children[6]. More recently, either emphysema[7] or cirrhosis[8], or both[9], have been reported in adults and elderly patients with A_1AT deficiency. In most instances, it has been postulated that the basic defect is the enzyme deficiency, whereas the disease processes represent secondary manifestations[10,11]. However, the possibility of the cirrhosis being the primary defect with subsequent appearance of the enzyme deficiency and resulting emphysema has never been explored. The purpose of our study is to test this hypothesis by initially determining the extent of the association between cirrhosis and emphysema.

9. Details of experimental design and procedures (append extra pages as necessary)

Lungs from consecutive autopsies of patients with cirrhosis will be included in the study. After careful removal, inflation and fixation will be accomplished with formaldehyde vapor by means of a modified Weibel's apparatus[12].

Low intensity roentgenograms will be performed on high contrast industrial films in a Faxitron machine after which tantalum bronchograms will be performed. Lungs will then be cut parasagitally at 2 cm. intervals with an electric knife. Sections will be examined with a zoom stereoscopic dissection microscope and interesting areas will be photographed.

In the sections of inflated specimens it will be possible to classify emphysema anatomically as centrilobular, panlobular, paraseptal, and irregular, according to the criteria of the American Thoracic Society[13]. A detailed assessment of each lung will determine certain parameters of the emphysema present such as distribution of the process within the lung, localization of the lesions within the secondary lobules and degree of pulmonary involvement.

Hepatic cirrhosis will be studied pathologically in the same autopsied patients by gross and histopathological techniques to include H and E and other stains such as Masson's trichrome, P.A.S., Snook's reticulin and others. Cirrhosis of the liver will be classified according to the criteria set forth by Rubin and Popper as: regular, mainly monolobular cirrhosis (Laennec's or nutritional cirrhosis) and irregular, mainly multilobular cirrhosis (postnecrotic or posthepatitic cirrhosis)[17]. The incidence of emphysema among all cirrhotic patients in general and the specific types of cirrhosis will then be statistically determined. Correlation will be made between the different types and degrees of emphysema associated with the various types of liver cirrhosis taking into account the smoking habits of the patients. Special attention will be paid to comparing the various specimen x-rays with the in vivo chest roentgenograms in an attempt to determine if the emphysema present could be diagnosed radiographically. In vivo and postmortem serum trypsin inhibitory capacity (S.T.I.C.) will be determined according to Hammarsten in the same patients whose lungs and liver are included in the study[15]. The S.T.I.C. values will then be correlated with the respective degrees of emphysema and cirrhosis. In addition, all sera will be phenotyped as to the exact level of A_1AT.

10. Space and facilities available (when elsewhere than item 2 indicates, state location):

For the study of lung and liver tissues the project will have the support of the Histopathology Laboratory of the Pathology Service at Hermann Hospital. For the special study of inflated and fixed lung specimens floor space will be made available in the Department of Pathology of the University of Texas Medical School at Houston, Texas. Facilities will also be provided by the Department of Pathology for the study of the in vivo chest roentgenograms and the various specimen Xrays. Serum trypsin inhibitory capacity determinations will be done at the Clinical Pathology Laboratory of Hermann Hospital.

Dr. Warren C. Miller and Dr. Donald C. Cannon are co-investigators in the project. Dr. Warren C. Miller will cooperate with us in identifying patients to enter the study. Dr. Donald C. Cannon will cooperate with us in determinationn of serum trypsin inhibitory capacity and phenotyping of the sera.

11. Additional facilities required:

The proposed program does not require collaboration with other institutions. Nevertheless, potential cooperation is available from the institutions where the principal investigator has previously been trained. Specifically, a large series of emphysematous lungs studied by the whole organ section technique is available to us at a Research Center in Florida. The incidence and patterns of cirrhosis could easily be determined among these emphysematous cases.

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12. Biographical sketches of investigator(s) and other professional personnel (append):

13. Publications: (five most recent and pertinent of investigator(s); append list, and provide reprints if available).

14. First year budget:

A. Salaries (give names or state "to be recruited") Professional (give % time of investigator(s) even if no salary requested)	% time	Amount
Technical (To be Recruited)	100%	$9,000.00
Sub-Total for A		9,000.00

B. Consumable supplies (by major categories)

1. Xray films	$800.00
2. Miscellaneous glassware	200.00
3. Reagents and chemicals	600.00
Sub-Total for B	1,600.00

C. Other expenses (itemize)

1. Analytic testing by Clinical Laboratory	$500.00
Sub-Total for C	500.00
Running Total of A + B + C	$11,100.00

D. Permanent equipment (itemize)

1. Specimen Inflating apparatus	$1,000.00
2. Base-sledge whole organ microtome	5,000.00
3. Faxitron specimen Xray machine	1,000.00
4. Stereozoom dissecting microscope	900.00
Sub-Total for D	7,900.00
E	1,665.00
Total request	$20,665.00

E. Indirect costs (15% of A+B+C)

15. Estimated future requirements:

	Salaries	Consumable Suppl.	Other Expenses	Permanent Equip.	Indirect Costs	Total
Year 2	$10,000	$2,500	$2,500		$2,250	$17,250
Year 3						

16. Other sources of financial support:
List financial support from all sources, including own institution, for this and related research projects.

CURRENTLY ACTIVE

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates

PENDING OR PLANNED

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates
1. Experimental Bleomycin Pulmonary Toxicity	University of Texas Health Sciences Center Institutional Grant	$5,000	1-1-76 -- 12-31-77

It is understood that the investigator and institutional officers in applying for a grant have read and accept the Council's "Statement of Policy Containing Conditions and Terms Under Which Project Grants Are Made."

Principal investigator

Typed Name Carlos W. M. Bedrossian, M.D.

Signature Carlos Bedrossian III Date 6-23-75

Telephone REDACTED
Area Code Number Extension

Checks payable to
The University of Texas Health Science Center
at Houston (Medical School)

Mailing address for checks
P. O. Box 20036
Houston, Texas 77025

Responsible officer of institution

Typed Name G. C. Franklin

Title Vice President for Business Affairs

Signature G C Franklin Date 7-1-75

Telephone REDACTED
Area Code Number Extension

REFERENCES:

1. Foraker, A. G., Bedrossian, C.W.M., and Anderson, A.E. Jr.: Myocardial dimensions and proportions in pulmonary emphysema. Arch. Pathol. 90:344-347, 1970.
2. Anderson, A.E., Jr., Bedrossian, C.W.M. and Foraker, A.G.: Systemic blood pressure in subjects with and without emphysema. A mer. Rev. Resp. Dis. 103:576-578, 1971
3. Bedrossian, C.W.M.: Coexistent emphysema and bronchogenic carcinoma. Cancer Bulletin 24:102-107, 1972.
4. Kueppers, F.: Alpha-1-antitrypsin: physiology, genetics and pathology. Human Genetics 6:207, 1968.
5. Laurell, C.B. and Eriksson, S.: The electrophoretic alpha-1 globulin pattern of serum in alpha-1-antitrypsin deficiency. Scand. J. Clin. Lab. Invest. 15:132, 1963.
6. Sharp, H.L., Bridges, R.A., Krivit, W. and Freier, E.F.: Cirrhosis associated with alpha-1-antitrypsin deficiency: A previously unrecognized inherited disorder. J. Lab. Clin. Med. 73:934-939, 1969.
7. Tarkoff, M.P., Kueppers, F. and Miller, W.F.: Pulmonary emphysema and alpha-1-antitrypsin deficiency. Amer. J. Med. 45:220-228, 1969.
8. Berg, N.O. and Eriksson, S.: Liver disease in adults with alpha-1-aNtitrypsin deficiency. New Engl. J. Med. 287:1,264-1,266, 1972.
9. Gherardi, G.J.: Alpha-1-antitrypsin deficiency and its effect on the liver. Human Pathol. 2:173-175, 1971.
10. Eriksson, S.: Studies in alpha-1-antitrypsin deficiency. Acta Med. Scand. 177, Suppl. 432, 1965.
11. Sharp, H. A.: Alpha-1-antitrypsin deficiency. Hosp. Pract. 83, 1971.
12. Greenberg, S.D., O'Neal, R.M. and Jenkins, D.E.: A rapid method of inflation-fixation for the morphologic study of chronic pulmonary disease. Amer. J. Clin. Path. 41:658-662, 1964.
13. American Thoracic Society: chronic bronchitis, asthma, and pulmonary emphysema: A statement by the Committee on Diagnostic Standards for nontuberculous respiratory diseases. Amer. Rev. Res. Dis. 85:762, 1962.
14. Rubin, E. and Popper, H.: The evolution of human cirrhosis as deduced from observations in experimental animals. Med. 46:163, 1967.

CURRICULUM VITAE

NAME: Carlos Wanes Menino Bedrossian

PLACE AND DATE OF BIRTH: REDACTED

SOCIAL SECURITY NUMBER: REDACTED

CITIZENSHIP: REDACTED

HOME ADDRESS: REDACTED

OFFICE ADDRESS: Department of Pathology University of Oklahoma Health Sciences Center, P.O. Box 26901 Oklahoma City, Oklahoma 73190

MARITAL STATUS: REDACTED

CHILDREN: REDACTED

EDUCATION:

R B.A. and B.S. Colegio Moura Lacerda, Ribeirao Preto, Sao Paulo, Brazil.

R M.D., University of Sao Paulo, Ribeirao Preto Medical School, Ribeirao Preto, Sao Paulo, Brazil.

MILITARY SERVICE: Lieutenant, Medical Corps, Brazilian Air Force, 1968.

POST GRADUATE ACTIVITIES:

1968-1969, Chief of Laboratories, 4th Aerial Zone Hospital, Brazilian Air Force, Sao Paulo, Brazil, Volunteer Assistant, Departments of Pathology, Santa Casa, Paulista de Medicina and Juqueri Hospitals, Sao Paulo, Brazil.

1969-1971, Resident in Pathology and Research Fellow in Pulmonary Pathology, Baptist Memorial Hospital, Jacksonville, Florida, U.S.A.

1971-1972, Fellow in Anatomical Pathology, The University of Texas M.D. Anderson Hospital and Tumor Institute, Texas Medical Center, Houston, Texas, U.S.A.

1972-1973, Fellow in Diagnostic Cytopathology and Pulmonary Pathology, Baylor College of Medicine, and Chief Resident in Pathology, Ben Taub General Hospital Texas Medical Center, Houston, Texas, U.S.A.

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HOSPITAL APPOINTMENTS:

1973-1975: Assistant Pathologist and Director of Cytopathology, University Hospital, Veterans Administration Hospital, and Oklahoma Children's Memorial Hospital, The University of Oklahoma Health Sciences Center, Oklahoma City, Oklahoma.

1975-Present: Assistant Pathologist and Director of Cytopathology, Hermann Hospital, Texas Medical Center, Houston, Texas.

ACADEMIC POSITIONS:

1973-1975: Assistant Professor of Pathology, Colleges of Medicine and Dentistry, The University of Oklahoma Health Sciences Center, Oklahoma City, Oklahoma

Associate Professor and Chairman, Department of Cytotechnology, College of Allied Health Professions, The University of Oklahoma Health Sciences Center, Oklahoma City, Oklahoma

1975-Present: Assistant Professor of Pathology, University of Texas Medical School at Houston, Texas Medical Center, Houston, Texas

LICENSES AND CERTIFICATES:

1968 - Conselho Regional de Medicine, Sao Paulo, Brazil, License #13110
1970 - Educational Council for Foreign Medical Graduates, Certificate #099-654-6
1971 - State of Texas Board of Examiners in the Basic Sciences, Certificate #19391
1972 - Texas State Board of Medical Examiners, License #D-9356
1973 - Oklahoma State Board of Medical Examiners License #10019
1974 - American Board of Pathology, Board Certified in Anatomic and Clinical Pathology

PROFESSIONAL ORGANIZATIONS:

REDACTED

REDACTED

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FIELDS OF SCIENTIFIC INTEREST:

Diagnostic Cytopathology
Clinical-pathologic correlations in chronic obstructive pulmonary disease.
Examination of excised lungs by pathological and radiological methods.
Electron microscopy of cancer and other pulmonary diseases.
Heart-Lung inter-actions in normal and in disease.

HONORS AND AWARDS:

1966, Academic Merit Diploma, The University of Sao Paulo, Ribeirao Preto, Sao Paulo, Brazil.

1967, Best Student in the Basic Sciences Award, The University of Sao Paulo, Ribeirao Preto, Sao Paulo, Brazil.

1972, Annual Clinical Training Research Project Award, The University of Texas M.D. Anderson Hospital and Tumor Institute, Houston, Texas.

1974, "Who's Who in Texas", United States Public Relations Service, Atlanta, Georgia.

ELECTED OFFICE:

President, Oklahoma Society of Cytopathology. 1974-75.

POST GRADUATE STUDIES:

Pathology of the Lung. University of California, San Diego, La Jolla, California. Averyll A. Liebow, Director, June 23-28, 1969.

Gynecologic and Obstetric Pathology, Armed Forces Institute of Pathology, Washington, D.C., Henry J. Norris, Director, October 26-30, 1970.

Dermal Pathology. The University of Texas M. D. Anderson Hospital and Tumor Institute, Houston, Texas. J. Leslie Smith, Jr., Director, August 23-27, 1971.

Clinical Pathology of Respiratory Diseases. The Association of Clinical Scientists, Chicago, Illinois. William Sunderman, Director, November 3-5, 1972.

A Symposium on Diseases of the Chest, The Fleischner Society, Boston, Massachusetts, Leo G. Rigler, Director, May 26-29, 1973.

PRESENTATIONS:

1. "Myocardial Dimensions and Proportions In Pulmonary Emphysema" to Florida Academy of Sciences, Jacksonville University, Jacksonville, Florida, March 5, 1970.

2. "Bronchitis Cronica y Enfisema Pulmonar" to the Mexican Patholgists Association, Sequros Sociales Hospital, Mexico City, Mexico, June 19, 1970.

3. "An Eclectic Approach to Lung Morphology" to the Brazilian Pathologists Association, Northeast Chapter, Recife, Pernamuco, Brazil, January 14, 1972.

4. "Interstitial Pneumonitis Associated with Bleomycin Therapy" to the International Academy of Pathology, Cincinnati, Ohio, March 15, 1972.

5. "Lung Cancer and Emphysema" to the Texas Chapter American College of Chest Physicians, San Antonio, Texas, May 13, 1972.

6. "Bleomycin Pulmonary Toxicity In Clinical and Experimental Subjects" to the Medical Research Division, Bristol Laboratories, Syracuse, N.Y., May 25, 1973.

7. "Ultrastructure of the Lung in Loeffler's Pneumonitis" to the Latin-American Pathologists Association, Merida, Yucatan, Mexico, November 27, 1973.

8. "The Lung in Cystic Fibrosis of the Pancreas" to the Latin-American Pathologists Association, Merida, Yucatan, Mexico, November 27, 1973.

9. "Electron Microscopic Changes and some Experimental Aspects of Bleomycin Pulmonary Toxicity" to the National Cancer Institute, Division of Cancer Treatment, NIH, Bethesda, Maryland, February 15, 1974.

10. "Esophageal Cytopathology: Review of Experience and Presentation of Interesting Cases" to the Texas Society of Cytology, Dallas, Texas, April 27, 1974.

11. "The Role of Cytopathology in the Diagnosis of Gastroesophageal Diseases" to the Oklahoma Society of Cytopathology, Oklahoma City, Oklahoma, May 15, 1974.

12. "Experimental Bleomycin Pulmonary Toxicity" to the Brazilian Society of Pathology, Curitiba, Parana, Brazil, September 4, 1974.

PRESENTATIONS: (continued)

13. "Ultrastructure of Human Bronchiolo-alveolar Carcinoma" to the Brazilian Society of Pathology, Curitiba, Parana, Brazil, September 7, 1974.

14. "Pitfalls in Diagnosing, Cell Typing and Judging Degree of Differentiation of Bronchogenic Carcinoma in Brush Cytology Specimens" to the American Society of Cytology, New York, New York, November 8, 1974.

PUBLICATIONS

1970: 1. Bohm, G.M., Bedrossian, C.W.M., De Luca, F. and Kerr, W.: The failure of nucleic acids to prevent the destruction of neurons in Chagas disease. Rev. Lat. Amer. Pathol. 9:17-23 (Mexico).

2. Foraker, A.D., Bedrossian, C.W.M., and Anderson, A.E.: Myocardial dimensions and proportions in pulmonary emphysema. Arch. Pathol. 90:344-347.

1971: 3. Anderson, A.E., Bedrossian, C.W.M., and Foraker, A.G.: Systemic blood pressure in subjects with and without emphysema. Amer. Rev. Resp. Dis. 103:576-578.

4. Bedrossian, C.W.M., Anderson, A.E. and Foraker, A.G.: Comparison of methods for quantitating bronchial morphology. Thorax 24:406-408, (London).

1972: 5. Luna, M.A., Bedrossian, C.W.M., Lichtiger, B. and Salem, P.: Interstitial pneumonitis associated with Bleomycin therapy. Amer. J. Clin. Pathol. 58:501-510.

6. Bedrossian, C.W.M.: Coexistent emphysema and bronchogenic carcinoma. Cancer Bulletin 24:102-107.

1973: 7. Bedrossian, C.W.M. and Martin, J.E.: Xeroradiography of the lung. Radiol. 107:217-218.

8. Bedrossian, C.W.M., Greenberg, S.D. and Duran, B.S.: Bronchial gland measurements: A continuing search for a yardstick. J. Exp. & Molec. Pathol. 18:219-224,

9. Bedrossian, C.W.M., Luna, M.D., MacKay, B. and Lichtiger, B.: Ultrastructure of pulmonary Bleomycin toxicity, Cancer 32:44-51.

10. Wurlitzer, F., Bedrossian, C.W.M., Ayala, A.G. and McBride, C.: Problems in diagnosing and treating infiltrating lipomas. Amer. Surg. 39:240-243.

1974: 11. Stork, W.J., Greenberg, S.D. and Bedrossian, C.W.M.: Fatal sarcoidosis. VI Int. Conf. on Sarcoidoses, Tokio, Japan. pp 462-472, Univ. Park Press.

12. Bedrossian, C.W.M. Ultrastructural changes and some experimental aspects of Bleomycin pulmonary toxicity. In "New Drug Seminar" sponsored by National Cancer Institute, Division of Cancer Treatment, Bethesda, Md., p. 169, Feb.

13. Bedrossian, C.W.M.: Current data regarding Bleomycin-induced pulmonary toxicity: An Audio Review. Intramed. Communications. In New York, N.Y., Vol. 2, Side 2, April.

1975: 14. Bedrossian, C.W.M., Greenberg, S.D., and Williams, L.J.: Ultrastructure of the lung in Loeffler's pneumonitis. Amer. J. Med., 58:438-443.

15. Bedrossian, C.W.M., Greenberg, S.D., Singer, D., Jenson, J., and Rosenberg, H.: The lung in cystic fibrosis of the pancreas. Human Pathology (In press).

1975 continued

16. Weilbaecher, D.G., Bedrossian, C.W.M., Greenberg, S.D. and Bentick, D.C.: Ultrastructure of human bronchioloalveolar carcinoma. Cancer (In press).

17. Bedrossian, C.W.M. and Rybka, D. L.: Bronchial brushing during fiberoptic bronchoscopy for the cytodiagnosis of lung cancer. Comparison with sputum and bronchial washings. Acta Cytologica. (Submitted for publication).

18. Bedrossian, C .W.M., Yawn, D.H., Greenberg, S.D. and O'Neal, R.M.: Experimentally-induced Bleomycin pulmonary toxicity in the pheasant - Part I: Histopathological findings. Lab. Invest. (Submitted for publication).

19. Bedrossian, C.W.M., Greenberg, S.D., Yawn, D.H. and O'Neal, R.M.: Experimentally-induced Bleomycin pulmonary toxicity in the pheasant - Part II: Ultrastructural observations. Lab. Invest. (Submitted for publication.)

1975 Work in Progress

1. Bedrossian, C.W.M., Greenberg, S.D., Luna, M.A., Skinner, F.M. and Jenkins, D. E.: Pathological patterns of chronic obstructive pulmonary disease associated with "environmental" and "endogenous" lung cancer. (In preparation).

Date: November 5, 1974

CURRICULUM VITAE

A. Personal Information:

Name in Full	Donald Charles Cannon
Business Address	University of Texas Medical School-Houston 6400 W. Cullen Blvd. Houston, Texas 77025
Business Phone	(713) 792-4701
Home Address	REDACTED
Home Phone	
Date of Birth	
Place of Birth	
Citizenship	
Marital Status	
Spouse's First Name	REDACTED
Number of Children	
Social Security Number	

B. Education

High School	William Chrisman High School, Independence, Missouri, R
College or University	Harvard College, Cambridge, A.B. R
	University of Chicago, Ph.D., R
Medical School	Washington University School of Medicine, St. Louis, R
	University of Chicago Medical School, M.D., R
Internship	UCLA Hospital, 1960-61, Pathology
Residencies	University of Chicago Clinics, July 1961-June, 1964-Pathology
Fellowships	Summer Fellow in Pathology, 1958, Washington University
	U.S.P.H.S. Pre-doctoral Trainee, 1960, University of Chicago, Immunopathology
	U.S.P.H.S. Post-doctoral Trainee, 1961-1963, University of Chicago, Immunopathology
Honors and Awards	Harvard College Scholarship, 1952-56
	Phi Beta Kappa, Harvard College, 1956
	Jackson Johnson National Scholarship, Washington University, 1956-1958
	President, first year medical class, Washington University

Honors and Awards
Chicago University Scholarship, 1959-1960
Alpha Omega Alpha, University of Chicago
Sigma Xi, University of Chicago
Markle Scholar in Academic Medicine, 1967-1968
Award of Merit for Distinguished Service in Advancing the Cause of Blood Banking, American Association of Blood Banks, 1967

Licensure
Missouri, 1962 (certificate 28946)
New York, 1965 (certificate 95632)
California, 1970 (certificate C-32725)

Board Certification
American Board of Pathology (clinical and anatomic), 1967

C. Professional Background:

Academic Appointments

Instructor of Pathology, University of Chicago, 1963-64
Instructor of Clinical Bacteriology and Pathology, University of North Carolina, 1964-65
Assistant Professor of Pathology, Upstate Medical Center, 1965-68
Associate Professor (in absentia), Upstate Medical Center, 1969
Visiting Professor of Pathology, The University of New Mexico, 1972-present
Associate Clinical Professor of Pathology, University of Southern California, 1973-present

Specific teaching responsibilities

Autopsy case study for sophomores, 1960-61
Teaching in general histopathology for sophomores, 1961-62
Lectures in liver pathology for sophomores and in endocrine pathology for seniors, 1963-64
Lectures in general pathology and autopsy case study for sophomores. Lectures and laboratory training in clinical bacteriology, autopsy and surgical pathology training for pathology resident physicians, 1964-65
Lectures in immunology, blood banking, and clinical chemistry; conference leader in general pathology and clinical pathology; and gross autopsy review for sophomores. Lectures to medical technology students and blood bank technology students. Clinical pathology training of pathology resident physicians. 1965-68.

Specific teaching responsibilities

Teaching in blood banking and immunology, Foundation for Advanced Education in the Sciences, National Institutes of Health, 1968-69
Director of Resident Training, Clinical Pathology Department, NIH, 1969-70
Lectures and conference teaching in acid-base and electrolytes for sophomores, 1972-74

Specific administrative responsibilities

Assistant Director and Assistant Attending Pathologist, Division of Clinical Pathology, Upstate Medical Center, 1965-68
Hospital Emergency Room - Outpatient Committee, 1965-68
Hospital Safety Committee, 1965-68; Chairman, 1967-68
Syracuse Red Cross Blood Program Committee, 1966-68
Hospital Disaster Planning Committee, 1967-68
House Officers and Graduate Students Committee, 1967-68
Syracuse Red Cross Blood Recruitment Committee, 1967-68
Medical Occupations Advisory Committee, Manpower Development Training Program, 1967-68; Chairman, Subcommittee on Laboratory Training
Cancer Subcommittee, Onondaga County Medical Society, 1967-68
Blood Bank Inspector, Northeast District, American Association of Blood Banks, 1967-68
General Director and Coordinator, American Association of Blood Banks Pre-convention Workshop on Blood Component Therapy, 10/21/67
Workshop Committee, American Association of Blood Banks, 1967-68
Member, Medical Technology Workshops Committee, American Society of Clinical Pathologists, 1969-71
Member, Ad Hoc Review Committee for Advanced Traineeships, Division of Allied Health Manpower, NIH, 1969
Panel Member, Interagency Board of U. S. Civil Service Examiners for Washington, D.C., 1969-70

Military service

U. S. Public Health Service, 1968-70

Other employment or activity

Chief, Diagnostic Reagents Section, Laboratory of Blood and Blood Products, Division of Biologics Standards, National Institutes of Health, July - December, 1968
Clinical Pathologist, Clinical Chemistry Service, Clinical Pathology Department, Clinical Center, NIH, January - June, 1969
Assistant Section Chief, Clinical Chemistry Service, Clinical Pathology Department, Clinical Center, NIH, July 1969 - June, 1970
Assistant Director (1970 - 71), Director (1971 - 74), Bio-Science Laboratories, Van Nuys, California
Professor and Director, Program in Pathology, Univeristy of Texas Medical School at Houston, Houston, Texas (1974 - Present)

D. Society Memberships:

National

REDACTED

E. Consultantships:

None

F. Research Activities:

Complete Bibliography

Appended.

Major Areas of Research Interest

Clinical chemistry, especially correlation of clinical laboratory tests with diagnosis.

Research in Progress

Correlation of serum triidothyronine assays with thyroid status.

Research grants in Past Five Years

None

10035459 12

BIBLIOGRAPHY

1. Cannon, D. C., and R. W. Wissler. Migration of Spleen Cells into the Blood Stream Following Antigen Stimulation of the Rat. Nature 207:654, 1965.

2. Ward, P. H., Cannon, D. C., and J. R. Lindsay. The Vestibular System in Multiple Sclerosis. A Clinical Histopathological Study. Laryngoscope 75:1031, 1965.

3. Cannon, D. C., and R. W. Wissler. Restoration of the Immune Response by Circulating Lymphocytes. Arch. Path. 83:188, 1967.

4. Cannon, D. C., and R. W. Wissler. Spleen Cell Migration in the Immune Response of the Rat. Arch. Path. 84:109, 1967.

5. Cannon, D. C., Immunoglobulin Analysis in Clinical Diagnosis. I. Quantitative Methods. Postgrad. Med. 46(2):55, 1969.

6. Cannon, D. C., Immunoglobulin Analysis in Clinical Diagnosis. II. Immunoelectrophoresis. Postgrad. Med. 46(3):55, 1969.

7. Cannon, D. C. Clinical Aspects of the Leukocyte Antibody Reaction. Postgrad. Med. 47:51, 1970.

8. Reed, A. H., Cannon, D. C., Winkelman, J. W., Bhasin, Y. P., Henry, R. J., and V. J. Pileggi. Estimation of Normal Ranges from a Controlled Sample Survey. I. Sex and Age-Related Influence on the SMA-12/60 Screening Group of Tests. Clin. Chem. 18:57, 1972.

9. Reed, A. H., Cannon, D. C., Pileggi, V. J., and J. W. Winkelman. Use of confidence intervals to assess precision of normal range estimates. Clin. Biochem. 6:29, 1973.

10. Winkelman, J. W., Cannon, D. C., Pileggi, V. J., and A. H. Reed. Estimation of norms from a controlled sample survey. II. Influence of body habitus, oral contraceptives and other factors on values for the normal range derived from the SMA 12/60 screening group of tests. Clin. Chem. 19:488, 1973.

11. Wybenga, D. R., Ibbott, F. A., and D. C. Cannon. Ionized calcium: Correction for pH and temperature effects. Accepted for publication in Clinical Chemistry.

12. Lowe, M. L., and D. C. Cannon. Improved Method for euglobulin clot lysis. Submitted for publication to Clinical Biochemistry.

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ABSTRACTS

1. Cannon, D. C. Cell Migration from the Rat Spleen in the Immune Response. Fed. Proc. 23:345, 1964.

2. Wybenga, D. R., Cannon, D. C., and Ibbott, F. A. Factor for Correcting Ionized Calcium Values Obtained from Serum Collected and Stored Without Special Precautions. Clin. Chem. 18:715, 1972.

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1. Fitch, R. W., Pierce, C., Hunter, R. L., Cannon, D. C., and R. W. Wissler. Recent Observations on the Origin and Fate of Antigen-Stimulated Cells in the Rat Spleen in Germinal Centers in Immune Responses, H. Cottier, N. Odartchenko, R. Schindler and C. C. Congden, Eds., Springer-Verlag, Inc., New York, 1967.

2. Cannon, D. C. Gastric and Duodenal Contents in Todd-Sanford Clinical Diagnosis by Laboratory Methods, I. Davidsohn and J. B. Henry, Eds., 14th ed., W. B. Saunders Co., Philadelphia, 1969.

3. Cannon, D. C. Examination of the Semen, Ibid.

4. Cannon, D. C., Olitzky, I. O., and J. A. Inkpen, Ch. 16, Proteins. In: Clinical Chemistry-Principles and Technics, R. J. Henry, D. C. Cannon and J. W. Winkelman, Eds., 2nd ed., Hoeber Division, Harper and Roe, Hagerstown, Maryland, May 1974.

5. Winkelman, J. W., Cannon, D. C., and S. L. Jacobs, Ch. 22, Liver Function Tests. In: (see No. 4 above).

6. Cannon, D. C., Ch. 30, Kidney Function Tests. In: (see No. 4 above).

7. Cannon, D. C., Ch. 31, Gastric Analysis. In: (see No. 4 above).

BOOKS

1. Henry, R. J., Cannon, D. C. and Winkelman, J. W., Editors. Clinical Chemistry-Principles and Technics, 2nd ed. Hoeber Division, Harper and Roe, Hagerstown, Maryland, May 1974.

BIBLIOGRAPHY (continued)

MISCELLANY

1. Lancaster, R. G., Cannon, D. C., Freeman, J., and R. Lehman. Atlas of Urine Sediment. The American Society of Clinical Pathologists, Chicago, 1970.

2. Laboratory Testing: Current state of the art.
Donald C. Cannon, M. D., Ph. D., Consultant
Patient Care, 21-67, June 30, 1972.

3. Laboratory Testing: Avoiding the pitfalls of office testing.
Donald C. Cannon, M. D., Ph. D., Consultant
Patient Care, 102-116, July 15, 1972.

4. How to gauge your lab's proficiency objectively.
Donald C. Cannon, M. D., Ph. D., Consultant
Patient Care, 82-95, September 15, 1972.

5. Cardiac Enzymes - Signposts to Diagnosis.
Donald C. Cannon, M. D., Ph. D., Consultant
Laboratory Management 12:27-29, February, 1974.

7. Cannon, D. C., Ch. 31, Gastric Analysis, In (see No. 4 above).

CURRICULUM VITAE

BIBLIOGRAPHY (continued)

NAME: Warren C. Miller, M.D.

BIRTHDATE: REDACTED

BIRTHPLACE: REDACTED

MARITAL STATUS: REDACTED

NO. OF CHILDREN: REDACTED

EDUCATION:

Medical School: University of Texas Southwestern Medical School
REDACTED (M.D. June 6, 1966)

Internship: Medical Internship
Wilford Hall USAF Hospital
REDACTED

Residency: Internal Medicine
USAF Medical Center Keesler
1967-1970

Fellowship: Pulmonary Medicine
Baylor College of Medicine
1972-1973

BOARD CERTIFICATION:

National Board of Medical Examiners - 1967
Diplomat, American Board of Internal Medicine-1971
Subspecialty Board in Pulmonary - 1974

MILITARY SERVICE: USAF Medical Corps - 1966-1972

TEACHING POSTS:

Assistant Chief, Pulmonary Division
USAF Medical Center Keesler, 1970-1971

Chief, Pulmonary Division
USAF Medical Center Keesler, 1971-1972

Assistant Instructor in Medicine
Baylor College of Medicine, 1972-1973

TEACHING POSTS, CONT'D:

Assistant Professor and Director, Pulmonary Division
University of Texas Medical School at Houston, 1973 - present

PROFESSIONAL SOCIETIES:

REDACTED

REDACTED

LICENSURE:

Texas - 1966
Louisiana - 1968
Florida - 1970

PUBLICATIONS:

Bogartz, L.J.; Miller, W.C.; Pulmonary Edama Associated with Propoxyphene Intoxication. JAMA, 215: 259, 1971.

Miller, W.C.; Toon, R: Mycobacterium Marinum in Gulf Fishermen. Arch Environ Health 27: 8, 1973.

Miller, W.C.; Toon, R.; Palat, H.; LaCroix, J.: Experimental Pulmonary Edema Following Re-expansion of Pneumothorax. Amer Rev Resp Dis 108: 664, 1973.

Hrnichek, G.; Skelton, J.; Miller, W.C.: Pulmonary Edema Associated with Salicylate Intoxication. JAMA: 230:866, 1974.

Miller, W.C.; Awe, R.; Effect of Nebulized lidocaine on Reactive Airways. Amer Rev Resp Dis: In Press.

Bowen, J.C; Miller, W.C.: Pathophysiologic Considerations in The Treatment of Posttraumatic Pulmonary Insufficiency. Amer J Surgery: In Press.

Miller, W.C.: Simi, W.W.: Clinical Use of Quiet Breathing Airway Resistance. Submitted for publication.

Simi, W.W; Miller, W.C: Effect of Aerosol Metaproterenol in Normal Subjects. In preparation.

Skelton, J; Cormia, F.; Miller, W.C.: Lobar Pulmonary Edema After Reexpansion of Pneumothorax. In preparation

ABSTRACTS:

Miller, W.C.; Stevens, P.M.: Spirographic prediction of combined restrictive and obstructive lung disease. Clin Research 22:47A, 1974.

Simi, W.W.; Miller, W.C.: Clinical use of quiet breathing airway resistance. Clin Research: 23:34A, 1975.

Miller, W.C.; Awe, R.: Effect of nebulized lidocaine on reactive airways. Clin Research: 23:34A, 1975.

THE COUNCIL FOR TOBACCO RESEARCH–U.S.A., INC.

July 7, 1975

Grant application No. 1044

PULMONARY

To: The committee comprising Drs. Gardner, Jacobson, Lynch and Wyatt

Subject: A. Sonia Buist, M.D., University of Oregon, School of Medicine, Portland, Oregon
New application No. 1044
"The role of alpha$_1$ antitrypsin deficiency as a risk factor in the development of chronic airways obstruction"

History

An informal inquiry was handled as Case No. 324 and encouraged.

Request

Application No. 1044 requests $22,561 for the first year of a three year project. Estimates for the second and third years are $24,883 and $27,321, respectively.

Document submitted (attached)

Application dated June 23, 1975 (11 pages, including C.V. of Dr. Buist).

D.S

DS:wg
Att.

#1044

THE COUNCIL FOR TOBACCO RESEARCH–U.S.A., INC.
110 EAST 59TH STREET
NEW YORK, N. Y. 10022
(212) 421-8865

Application for Research Grant
(Use extra pages as needed)

Date: 6/23/75

1. Principal Investigator (give title and degrees):

A. Sonia Buist, M.D.
Assistant Professor of Medicine and Physiology

2. Institution & address:

University of Oregon Health Sciences Center
School of Medicine
3181 S.W. Sam Jackson Park Road
Portland, Oregon 97201

3. Department(s) where research will be done or collaboration provided:

Department of Physiology

4. Short title of study:

The role of alpha$_1$ antitrypsin deficiency as a risk factor in the development of chronic airways obstruction.

5. Proposed starting date: January 1976

6. Estimated time to complete: 3 years

7. Brief description of specific research aims:

The specific aim of the proposed research is to examine the role of alpha$_1$ antitrypsin (α_1AT) deficiency as a risk factor in the development of chronic airway obstruction.

Cigarette smoking is the only factor known at present to bear a causal relationship to the development of chronic airway obstruction (1). Clearly, however, this is not a 1 to 1 relationship since all who smoke do not develop clinical or physiological evidence of impairment. Indeed, in a recent population survey carried out by the Collaborative Study on Smoking and Airways Obstruction* in which both spirometry and the closing volume test were used, the prevalence of physiologic impairment in smokers aged 25 to 54 from randomly selected populations in Portland, Montreal and Winnipeg, ranged between 15 and 25%. Furthermore, in this study, no dose response relationship was found between cigarettes and either closing volumes (closing volume (CV) expressed as a percent of vital capacity (VC) i.e. CV/VC, and closing capacity (CC) expressed as a percent of total lung capacity (TLC) i.e. CC/TLC) or the slope of the alveolar plateau of the single breath nitrogen test ($\Delta N_2/L$). (2) The conclusions drawn from this study were that factors other than smoking, such as environmental pollution, childhood infection, and/or genetic

* The Collaborative Study on Smoking and Airways Obstruction designates research carried out in Portland (A.S. Buist), Montreal (P.T. Macklem) and Winnipeg (R.M. Cherniack) under a contract from the National Heart and Lung Institute.

7. (continued)

and biochemical factors play an important and perhaps synergistic role in the development of airway obstruction.

It has been well documented that homozygous α_1AT deficiency (P_i phenotype Z2) is strongly associated with the development of severe panlobular emphysema at an early age (3). However, it has also been demonstrated that some persons with homozygous α_1AT deficiency do not develop disease (4). The relationship between a homozygous deficiency of α_1AT and the development of disease is thus not entirely clear. The relationship between the heterozygous state (P_i phenotype MZ) and the development of disease is even more controversial. Some investigators have suggested that heterozygotes are no more at risk of developing emphysema than are persons with normal levels of α_1AT (5). Other investigators have put forward the opposite point of view (6) which has been supported by a recent study in which the results of lung function studies performed on 54 adults of P_i type MZ were compared to those in 69 adults with P_i type MM(7). In this study, the MZ adults had significantly inferior function as measured by arterial oxygen tension, lung elastic recoil, maximal expiratory flow and closing capacity. In addition, cigarette smoking and P_i type MZ interacted additively and caused greater reduction in function than either factor produced independently.

Unfortunately, most of the studies on α_1AT deficiency have had to use highly selected populations such as patients and relatives of patients attending outpatient clinics or hospital inpatients. This has led to difficulties in the interpretation of findings and some confusion as to the true role of α_1AT deficiency, both complete and partial, in the development of chronic airway obstruction. Exactly what this role is assumes considerable importance because the prevalence of α_1AT heterozygosity (P_i phenotype MZ) has been estimated at 30 per 1000 of the newborn population in the United Kingdom (8) and also because α_1AT deficiency is to date the only known model of a genetically inherited condition which leads to the development of chronic airway obstruction, with the exception of cystic fibrosis. The aim of the proposed research is to try to clarify the role of α_1AT deficiency as a risk factor in the development of chronic airway obstruction by establishing a cohort of P_i type MZ heterozygotes, and following the cohort prospectively over a period of years with repeated lung function tests.

8. Brief statement of working hypothesis:

The hypotheses to be tested are that:

(i) MZ heterozygotes for α_1AT deficiency have impaired lung function as measured by a wide range of tests of lung mechanics.

(ii) The MZ heterozygote does not belong to the high risk category for the development of chronic airway obstruction unless there is some additive factor such as cigarette smoking, occupational exposure to pollutants or atopic history.

9. Details of experimental design and procedures (extra pages appended)

(i) Subjects:

Since 1971, neonatal screening for α_1AT deficiency has been carried out on cord blood samples from all newborns in Oregon. To date 22 infants with P_i type ZZ homozygous α_1AT deficiency have been detected and confirmed out of a total of 110,000 births, giving a gene frequency in Oregon of approximately 1 in 30 of the population. We propose to do family pedigrees on these infants and attempt to study all first degree relatives. Since the parents of the infants will mostly be young (in 20's and 30's), this will establish an excellent cohort for prospective study

(ii) Methods

(a) Spirometry:

Tests for the measurement of forced vital capacity (FVC), FEV_1 and $MMEF_{25-75\%}$ will be performed on all subjects using standardized spirometric methods as established for epidemiologic studies by the National Heart and Lung Institute (9). Tests will be performed on a Vitalograph wedge-type spirometer or Bennett Remac Pulmonary Function Testing Unit. Both instruments will be calibrated against a Collins Stead-Wells spirometer at regular intervals. Using the prediction equations of Morris et al.(10), an abnormal test value would be defined as a value which is > -2 SEE below the predicted value for age, sex and height.

(b) Single breath nitrogen test for measurement of closing volumes:

Closing volume, CC, TLC, RV and $\Delta N_2/L$ will be obtained from the SBNT for the measurement of closing volumes as standardized by the Collaborative Study on Smoking and Airways Obstruction (11). The equipment currently used for this test includes a rolling seal spirometer (Cardio Pulmonary Instruments), a nitrogen analyzer (Med-Science), XYY' Recorder (Hewlett Packard) and bag-in-box system.

(c) Lung mechanics:

Static pressure-volume (P-V) curves, and maximum expiratory flow-volume (MEFV) curves using air and an oxygen-helium (O_2-He) mixture for measurement of volume of isoflow ($\dot{V}isoV$) as described by

9. (ii) (c) continued

Hutcheon et al. (12) will be carried out using the standardized procedure proposed by the Collaborative Study on Smoking and Airway Obstruction (13). The equipment used for studies of lung mechanics will include an Ohio 3000 constant volume body plethysmograph to be used as an integrated-flow plethysmograph as described by Leith and Mead (14).

(iii) Experimental Design

Initially a family pedigree of the proband will be drawn up. All first degree relatives of the proband over the age of 18 will be contacted and invited to participate in the study. Testing will consist of

(1) P_i phenotyping
(2) Respiratory system questionnaire (expanded NHLI questionnaire).
(3) Spirometric tests for measurement of FVC, FEV_1, $MMEF_{25-75\%}$.
(4) Single breath N_2 test for measurement of CV/VC, CC/TLC, $\Delta N_2/L$, TLC and RV.
(5) Lung mechanics studies for measurement of static P-V curves, V iso $\dot{V}$, flow at 50% VC ($\dot{V}_{max50}$) and 25% VC ($\dot{V}_{max\ 25}$).

After the initial test occasion, subjects will be asked to return for repeat testing probably at 2 year intervals. It is our intention to make this a long term prospective study. Controls matched for age, sex and smoking habits will be drawn from a long term prospect study, currently in progress, of a randomly selected population of non-smokers, smokers and ex-smokers living in Oregon. This cohort was first studied in 1974.

Data will be analyzed to look for differences in initial lung function between MZ/and MM subjects controls and for differences in the time course of change of the various tests of lung function between the different groups. We plan to follow the control group until 1980, (6 years total). It would be realistic to contemplate following the α_1AT cohort for a similar length of time in order to provide sound longitudinal data from which valid conclusions can be drawn about the role of α_1AT deficiency as a risk factor in the development of chronic airway obstruction.

SIGNIFICANCE

The important contribution that this study can make that is not readily available from other groups studying α_1AT deficiency is that we have been able to obtain an unselected population of homozygotes (P_i type ZZ) for study, i.e. a population which is truly representative of the frequency of α_1AT deficiency in Oregon. This has been made possible because all newborns in Oregon since 1971 have been tested, thereby giving access to a large population of heterozygotes which has not been selected on the basis of symptomatology or disease, or because the subjects were attending an outpatient clinic. In this way, an accurate assessment of the lung function of heterozygotes can be obtained and the role of the α_1AT heterozygous state in the development of chronic airways obstruction can be more clearly defined.

REFERENCES

1. The health consequences of smoking. A report of the Surgeon General: 1974. DHEW

2. Buist, A.S., Macklem, P.T. and Cherniack, R.M.
Relationship between smoking and single breath nitrogen washout in Portland, Oregon, Winnipeg, Manitoba and Montreal, Quebec. To be presented at International Union Against Tuberculosis Meeting, Mexico City, Sept. 1975.

3. Guenter, C.A., Welch, M.H. and Hammarsten, J.F.
Alpha-1 antitrypsin deficiency and pulmonary emphysema. Annual Review of Medicine 22, 283, 1971.

4. Welch, M.H., Reinecke, M.E., Hammarsten, J.F. and Guenter, C.A.
Antitrypsin deficiency in pulmonary disease: The significance of intermediate levels. Ann. Intern. Med. 71, 533, 1969.

5. Mittman, C. Summary of Symposium on Pulmonary Emphysema and Proteolysis.
American Rev. Resp. Dis. 105, 431, 1972.

6. Lieberman, J.
Heterozygous and homozygous alpha$_1$-antitrypsin deficiency in patients with pulmonary emphysema.
New Engl. J. Med. 281, 279, 1969.

7. Cooper, D.M., Hoeppner, V., Cox, D. Zamel, N., Bryan, A.C., and Levison, H.
Lung function in alpha$_1$-antitrypsin heterozygotes (Pi type MZ).
American Review of Respiratory Dis. 110, 708, 1974.

8. Cook, P.J.L.
Genetic aspects of the Pi system. Postgrad. Med. J. 50, 362, 1974.

9. Recommended Standardized Procedures for NHLI Lung Program Epidemiology Studies, NHLI, 1971.

10. Morris, J.F., Koski, W.A. and Johnson, L.C.
Spirometric standards for healthy nonsmoking adults. Amer. Rev. Resp. Dis. 103, 57, 1971.

11. Suggested standardized procedures for closing volume determinations (Nitrogen Method), distributed by NHLI, 1973.

12. Hutcheon, M., Griffin, P., Levison, H., and Zamel, N.:
Volume of isoflow Amer. Rev. Resp. Dis. 110, 458, 1974.

13. Macklem, P.T.,
Procedures for standardized measurements of lung mechanics.
Distributed by NHLI, Nov. 1974.

14. Leith, D.E. and Mead, J.
Principles of body plethysmography available from National Heart and Lung Institute, Nov. 1974.

1003545925

10. Space and facilities available (when elsewhere than item 2 indicates, state location):

The facilities currently in use include a laboratory (725 sq. ft.), equipped for general research in respiratory physiology, an office (130 sq. ft.) occupied by Dr. Buist. All of these facilities are located in the Department of Physiology.

The major items of permanent equipment on hand include:

1. Ohio 3000 Body Plethysmograph System, modified to be used as an integrated flow plethysmograph.
2. Hewlett-Packard XYY' Recorder and Houston Instrument XY Recorder.
3. CPI 220 Rolling Seal Spirometer.
4. Med Science N_2 Analyzer.
5. Hewlett-Packard Programmable Computer 9810 and Plotter (shared).
6. Brush 2 Channel Recorder
7. Stead-Wells Spirometer.

Hermansen, L.F. and Buist, C.A.
Antitrypsin deficiency in pulmonary disease. The significance of intermediate levels. Ann. Intern. Med. 71, 533, 1969.

5. Mittman, C. Summary of symposium on [illegible] and proteinosis. American Rev. Resp. Dis. [illegible], 1971.

Heterozygous and homozygous alpha$_1$-antitrypsin deficiency in patients with pulmonary emphysema

11. Additional facilities required:

Rotameter (Fisher-Porter) for calibrating body plethysmograph for air and helium.

8. Cook, P.J.L.
[illegible]
NHLI, 1971.

12. Biographical sketches of investigator(s) and other professional personnel (append):

13. Publications: (five most recent and pertinent of investigator(s); append list, and provide reprints if available).

12. Biographical Sketch

Aline Sonia Buist, M.B., Ch.B., M.D.
Assistant Professor of Medicine and Physiology

PERSONAL:
Birthdate: REDACTED
Married: REDACTED
Children: R

EDUCATION:
R University of St. Andrews Medical School, M.B., Ch.B.
R Residency in General Practice and Internal Medicine, University of Colorado Medical School
R Fellowship in Pediatric Chest Disease, University of Oregon Medical School
R Bellinger Fellowship in Clinical Pulmonary Disease (part time)
R Fellowship in Pulmonary Physiology, University of Oregon Medical School

EXPERIENCE:

Teaching

1970-1972 Clinical Instructor in Medicine, University of Oregon Medical School
1970-1972 Clinical Research Associate, Dept. of Physiology, University of Oregon Medical School
1972-pres. Assistant Professor of Medicine, Division of Chest Diseases, University of Oregon Medical School
1975-pres. Assistant Professor of Medicine, Dept. of Physiology, University of Oregon Medical School

Extramural Boards, Committees, Consults., etc.

1970-1972 Medical Director, Emphysema Screening and Research Center, Portland, Oregon
1974-pres. NIH Study Section for Young Investigators Pulmonary Research Awards
Pulmonary Academic Awards Committee, National Heart Lung Institute
Committee of the Structure and Function Assembly, American Thoracic Society
1975-pres. Member of Cardiovascular and Respiratory Study Section, National Heart and Lung Institute

HONORS

M.D. with commendation, University of Dundee, 1973

SOCIETY MEMBERSHIPS

REDACTED REDACTED

10035459 27

13. PUBLICATIONS:

1. Buist, A.S. and Ross, B.B. Predicted values for closing volumes using a modified single breath nitrogen test. Amer. Rev. Resp. Dis. 107: 744, 1973.

2. Buist, A.S., Van Fleet, D.L., and Ross, B.B. A comparison of spirometric tests and the measurement of closing volumes in an Emphysema Screening Center. Amer. Rev. Resp. Dis. 107: 735, 1973.

3. Buist, A.S. Early detection of airways obstruction by the closing volume technique. Chest. 64: 495, 1973.

4. Buist, A.S. and B.B. Ross. Quantitative analysis of the alveolar plateau in early airway obstruction. Amer. Rev. Resp. Dis. 108: 1078, 1973.

5. Buist, A.S. Current Concepts: The single breath nitrogen test. New Engl. J. of Med. In press.

1970-1972 Medical Director, Emphysema Screening and Research Center,

Research Awards

Budget Justification

Salaries

Two half-time salaries are requested -- for a research associate with considerable experience with the sophisticated electronics entailed in body plethysmography (Mr. B.E. Adams) and one for a research assistant to track down the subjects, do family pedigrees, spirometry and reduction of data.

No salaries are requested for Dr. Buist and Dr. Sexton. Dr. Buist currently holds a Research Career Development Award from the National Heart and Lung Institute and Dr. Sexton holds a Postdoctoral Statistical Fellowship (NIH Grant GMS 01736).

Equipment

A rotameter is requested for calibrating the body plethysmograph for air and helium.

Years 2 and 3

A 10% increase is requested for all categories of the budget with the exception of the equipment category. $500 for years 2 and 3 are requested for the purchase or replacement of minor items of equipment. e.g. transducer for body plethysmograph, needle valve for N_2 analyzer, new pneumotach lead, etc.

14. First year budget:

A. Salaries (give names or state "to be recruited")		% time	Amount
Professional (give % time of investigator(s) even if no salary requested)			
A.S. Buist	Principal Investigator	25%	REDACTED
B.E. Adams	Research Associate	50%	
G. Sexton	Statistical Postdoctoral Fellow	20%	
Technical			
To be recruited	Research Assistant	50%	$4,500
Payroll assessment 15% includes FICA, Pers, SIAC, etc. = $2,025			$2,025
		Sub-Total for A	$15,525

B. Consumable supplies (by major categories)

Supplies for spirometry and single breath N_2 test, (O_2, calibrating gases, mouthpieces, etc.)	500
Supplies for body plethysmography (O_2, helium, esophageal balloons, mouthpieces, polaroid film, topical anesthesia, etc.)	500
Secretarial supplies, sterilizing materials, recording paper, etc.	500
Sub-Total for B	$ 1,500

C. Other expenses (itemize)

Computer costs (keypunching @ $6/hr, programming @ $9/hr and computer time @ $300/hr)	1,000
Service and repair of equipment	1,000
Postage, telephone, xerox	250
Sub-Total for C	$ 2,250
Running Total of A + B + C	$19,275

D. Permanent equipment (itemize)

Rotameter	395
Sub-Total for D	$ 395
E	$ 2,891
Total request	$22,561

E. Indirect costs (15% of A+B+C)

15. Estimated future requirements:

	Salaries	Consumable Suppli	Other Expenses	Permanent Equip.	Indirect Costs	Total
Year 2	17,078	1,650	2,475	500	3,180	24,883
Year 3	18,785	1,815	2,723	500	3,498	27,321

10035459930

16. Other sources of financial support:
List financial support from all sources, including own institution, for this and related research projects.

CURRENTLY ACTIVE

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates
Smoking and Chronic Airways Obstruction	NIH-NHLI Contract N01-HR3-2900	$209,472	September 1972 - August 1975
Research Career Development Award	NIH-NHLI 1 K04 HL00115	$ 25,000	February 1975 - January 1976

PENDING OR PLANNED

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates
Natural history of chronic airways obstruction*	NIH-NHLI	$347,275	September 1975 - August 1980
Smoking and Chronic Airways Obstruction*	NIH-NHLI Renewal of Contract N01-HR3-2900	$354,938	September 1975 - August 1979

* If awarded the contract, the grant application will be withdrawn.

It is understood that the investigator and institutional officers in applying for a grant have read and accept the Council's "Statement of Policy Containing Conditions and Terms Under WRRA Project Grants Are Made."

Principal investigator

Typed Name A. Sonia Buist

Signature [signature] REDACTED Date 6/23/75

Telephone ___ Area Code ___ Number ___ Extension ___

Checks payable to

University of Oregon Health Sciences Center

Mailing address for checks

3181 S.W. Sam Jackson Park Road

Portland, Oregon 97201

Responsible officer of institution

Typed Name Lewis W. Bluemle, Jr., M.D.

Title President

Signature [signature] Date 6-25-75

Telephone ___ Area Code REDACTED Number ___ Extension ___

10035459931

THE COUNCIL FOR TOBACCO RESEARCH–U.S.A., INC.

July 7, 1975

Grant application No. 1043

PULMONARY

To: The committee comprising Drs. Gardner, Jacobson, Lynch and Wyatt

Subject: Hugh E. Evans, M.D., Jewish Hospital and Medical Center, Brooklyn
New application No. 1043
"Relationship of non-MM phenotypes and lung disease among infants"

History

The applicant took the option to submit application without consideration as a case.

Request

Application No. 1043 requests $35,357 for the first year of a two year project.

Documents submitted (attached)

1. Application dated June 25, 1975 (10 pages including C.V.s of Drs. Evans and Yong Ho Shin.

2. Four reprints and one manuscript.

D.S.

DS:wg
Att.

#1043

THE COUNCIL FOR TOBACCO RESEARCH–U.S.A., INC.
110 EAST 59TH STREET
NEW YORK, N.Y. 10022
(212) 421-8885

RECEIVED
JUN 30 1975

Application for Research Grant
(Use extra pages as needed)

Date: June 25, 197

1. Principal Investigator (give title and degrees):

Hugh E. Evans, M.D.
Director, Department of Pediatrics
Jewish Hospital and Medical Center of Brooklyn

Professor of Pediatrics
Downstate Medical Center
Brooklyn, New York

2. Institution & address:

Jewish Hospital and Medical Center of Brooklyn
555 Prospect Place
Brooklyn, New York 11238

3. Department(s) where research will be done or collaboration provided:

Department of Pediatrics
Jewish Hospital and Medical Center of Brooklyn

4. Short title of study:

Relationship of non-MM phenotypes and lung disease among infants.

5. Proposed starting date: January 1, 1976

6. Estimated time to complete: Two Years

7. Brief description of specific research aims:

This study is designed to screen newborn infants for non-MM phenotypes of alpha-1-antitrypsin, to correlate the frequency, severity and type of lung disease observed in the first year and one half of life with these phenotypes, to evaluate the fertility of mothers with non-MM newborns and to contrast the biochemical and physical characteristics of the MM phenotype in newborns with those seen in infants. The questions of this investigation are: Is a newborn infant more likely to develop croup, bronchiolitis, asthma, pneumonia or other lung disease if he is of a non-MM than MM phenotype? Furthermore, are there ethnic predispositions to both the non-MM phenotype and to resultant lung disease. If non-MM phenotypic infants are at greater risk of lung disease, are there environmental control measures which could be selectively applied to mitigate these illnesses? If mothers of non-MM phenotype newborns have inherently greater fertility than those of the MM phenotype, would this have implications for family planning studies? If the MM pattern of newborn infants differs from the MM protein seen in childhood does this offer important clues regarding molecular structure?

8. Brief statement of working hypothesis:

Non-MM phenotypes may play a major role in the pathogenesis of common, severe respiratory diseases of infants. This may be particularly true in crowded, environmentally adverse conditions typical of the ghetto population we serve. Furthermore, there may be ethnic determinants, as suggested in emphysema among adults. Perhaps infants with non-MM phenotypes have an imbalance between proteolytic enzymes derived from bacteria, leukocytes or alveolar macrophages and serum inhibitory capacity. Screening of newborn infants may be a practical approach to identification of those at high risk for development of subsequent lung disease. Environmental control may mitigate pulmonary disorder in such cases.

9. Details of experimental design and procedures (append extra pages as necessary)

Enrollment period: Umbilical cord sera Pi phenotyping will be obtained following each normal full term delivery at the Jewish Hospital and Medical Center of Brooklyn (JHMCB) from January 1, 1976 to July 1, 1976. Based on earlier experience we would anticipate that 1,000 infants will be included and that 80 of these will have a non-MM phenotype. Phenotyping will be done by crossed antigen-antibody electrophoresis, originally described by Fagerhol and Laurell,[1,2] or by isoelectrofocusing Quantitation of serum inhibitor will be carried out with radial immunodiffusion[3] and the antitrypsin activity test of Erlanger.[4] Each non-MM infant will be matched randomly for date of birth, sex and race with an MM newborn for purposes of subsequent follow-up.

Evaluation period: Over an 18 to 24 month interval each of the non-MM and control cases will be evaluated from a clinical point of view. They will receive their "well-baby" care in the clinics devoted to that purpose at the JHMCB. They will also be treated for all illnesses, respiratory or otherwise, and admitted to the ward as clinical judgment dictates. Every 3 months their hospital records, and the records of visits to their private physicians will be analyzed for the following:

1. Episodes of all illness.
2. Episodes of all respiratory illness.
3. Specific respiratory tract diagnosis, including chest x-rays, CBC, blood gases, bacterial culture results.
4. Hospitalizations, number, duration, discharge diagnosis; laboratory data as in #3.
5. Growth and development at age 1 and 2 years.

The data derived from each of the 2 groups will be compared to determine if there is a difference in the frequency of respiratory or other illness between the MM and the non-MM groups. Family counselling, based on medical knowledge is not possible, at present. Indeed information derived from the follow-up of non-MM infants may form the basis for such advice in the future.

continuation question #9

Phenotypes will be obtained on the mothers, and where possible, the fathers of non-MM newborns. A detailed reproductive history will be obtained and compared with that of a randomly selected control population of mothers of MM newborns. This will include the number of spontaneous abortions, induced abortions, living children and total pregnancies. The history will also include use of contraceptive techniques if any. Pending analysis of the data it may not be appropriate to offer family counseling to the non-MM group.

An additional laboratory study will be a comparison of the temperature stability of MM phenotypes in newborns with those of children. Sialic acid levels using the assay of Warren[5] and sialyltransferase levels as determined by Kuhlenschmidt et al[6] will be measured in each serum. Our previous studies suggest that the biosynthesis of alpha-1-antitrypsin may be incomplete at birth. The MM pattern of newborns, in our study, has a more cathodal mobility which is different from that seen in children. The sialic acid component may be the basis for phenotypic distinction, as suggested by Cox[7] and by Bell and Carrell,[8] and could contribute to the variation in the MM pattern typical of newborn infants.

question #9 (continued)

References for Laboratory Methods

1. Fagerhol, M.K. The Pi-system: Genetic variants of serum alpha-1-antitrypsin. Ser. Haematal 1: 153-161, 1968

2. Fagerhol, M.K. and Laurell, C-B. The polymorphism of "pre-albumins" and alpha-1-antitrypsin in human sera. Clin. Chem. Acta 16:199, 1967

3. Mancini, M., Carbonara, A. and Heremans, F. Immunochemical quantitation of antigens by single radial immunodiffusion. Immun. Chem. 2:234, 1965

4. Erlanger, B.F., Kokowsky, N and Cohen W. The preparation and properties of two new chromogenic substrates or trypsin. Arch. Biochem. 95:271, 1961

5. Warren, L. The thiobarbituric acid assay of sialic acids. J. Biol. Chem. 8:1971-1975, 1959

6. Kuhlenschmidt, M.S. et al. Demonstration of sialyltransferase deficiency in the serum of a patient with alpha-1-antitrypsin deficiency and hapatic cirrhosis. Lab. Inves. 4:413-419, 1974

7. Cox, D.W. The effect of neuraminidase on genetic variants of alpha-1-antitrypsin. Am. J. Hum. Gen. 27:165-177, 1975

8. Bell, O.F. and Carrell, R.W. Basis for the defect in alpha-1-antitrypsin. Nature 234:410-411, 1973 (June 15)

10. Space and facilities available (when elsewhere than item 2 indicates, state location):

The newborn service at Jewish Hospital and Medical Center of Brooklyn is one of the largest in the borough with over 2700 deliveries annually. We have previously had the complete cooperation of the Department of Obstetrics, Dr. Morton Schiffer, Director and would again in the proposed study. The Pediatric Out Patient Department and In Patient units are fully staffed and equipped to carry out the proposed studies. The Loewe Laboratory has carried out the proposed tests of alpha-1-antitrypsin for the past 1½ years. Refrigerators, centrifuges, electrophors, and the usual laboratory reagents and supplies are available.

11. Additional facilities required:

None

12. Biographical sketches of investigator(s) and other professional personnel (append):

13. Publications: (five most recent and pertinent of investigator(s); append list, and provide reprints if available).

question #12

Dr. Hugh E. Evans REDACTED REDACTED REDACTED He graduated from Columbia College, R cum laude, and Downstate Medical School, R His Internship and Residency were at Johns Hopkins Hospital, 1958-60, 1962-63. He was a Clinical Associate in the National Institute of Allergy and Infectious Diseases, N.I.H., Bethesda, Maryland, 1960-62. He was Associate Director of Pediatrics, Harlem Hospital Center and Associate Clinical Professor of Pediatrics, Columbia University, 1966-73. Presently he is Professor of Pediatrics, Downstate Medical Center and Director of Pediatrics, Jewish Hospital and Medical Center of Brooklyn, Memberships include REDACTED REDACTED REDACTED interests include the role of alpha-1-antitrypsin deficiency in neonatal lung disease and factors influencing the neonatal bacterial flora. He is senior author of the textbook, "Perinatal Medicine," which is in press for October, 1975.

Dr. Yong Ho Shin REDACTED He graduated from Pusan National University in R and from the Pusan National University School of Medicine in R He spent 4 years as a physician in the South Korean Army, the last 2 of which were in a Tuberculosis Hospital in Masan, Korea. His Internship, in this country was at Christ Hospital, Jersey City, New Jersey (1969) and he was a first year Resident in Martland Hospital, Newark, New Jersey in 1970. Following this he was a Senior Resident and a Fellow in Pulmonary Disease at Harlem Hospital Center, 1971-June 1973. He completed his training in Pulmonary Disease at the Jewish Hospital and Medical Center of Brooklyn in June 1974. He is a full-time Attending, in charge of Pulmonary Disease at JHMCB, and a Clinical Instructor in Pediatrics at Downstate Medical Center. He has been an active participant in the studies outlined.

question #13 (continued)

1. Evans, H.E., Mandl, I. and Glass, L. Serum Enzyme Inhibitors, Immunoglobulins and Upper Respiratory Tract Bacteria in Asthma. Am. Rev. Resp. Dis. 117:416-418, 1971 (October)

2. Mandl, I., Keller, S., Fierer, J.A. and Evans, H.E. The Role of Proteolytic Enzyme Inhibitors and Connective Tissue Proteins in the Maturation of the Lung. Harvard Conference on Respiratory Distress Syndrome. Academic Press, 99-115, 1973

3. Evans, H.E., Keller, S. and Mandl, I. Lung Tissue Elastin Composition in Newborn Infants with the Respiratory Distress Syndrome and Other Diseases. Journal of Clinical Investigation. J. Clin. Invest. 54:213-217, July, 1973

4. Fierer, J., Mandl, I. and Evans, H.E. Alpha-1-antitrypsin in the Lungs of Newborns with Respiratory Distress Syndrome. J. Ped. 85:698-701, Nov. 1974

5. Evans, H., Formaini, N. and Mandl, I. Prevalence of Pi types among newborns of different ethnic backgrounds. Protides of Biological Fluids 23rd Colloquium H.P. Peeters, ed. Pergamon Press, 1976

14. First year budget:

A. Salaries (give names or state "to be recruited")	% time	Amount
Professional (give % time of investigator(s) even if no salary requested)		
Dr. Hugh E. Evans	25%	R
Dr. Yong Ho Shin	50%	R
Technical		
Miss Nora Formaini	90%	R
Miss Lynn Perrott	90%	R
Sub-Total for A		REDACTED

B. Consumable supplies (by major categories)

1. Material for isoelectric focusing.	1,295
2. Material for crossed antigen-antibody electrophoresis.	2,480
3. Material for antitrypsin activity testing.	170
4. Material for radial immunodiffusion.	400
(See detailed list appended) Sub-Total for B	$4,345

C. Other expenses (itemize)

Sub-Total for C	
Running Total of A + B + C	30,745

D. Permanent equipment (itemize)

Sub-Total for D	
E. Indirect costs (15% of A+B+C) E	4,612
Total request	$35,357

15. Estimated future requirements:

	Salaries	Consumable Suppl.	Other Expenses	Permanent Equip.	Indirect Costs	Total
Year 2	REDACTED	$4,649			$4,935	$37,832
Year 3						

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question #14b (continued)

1.	ampholines	$360
	complete set including buffer tanks cooling plates tubing leads flow meter	890
	acrylimide gel BIS (N, N^1-Methylene-bis-acrylimide) TEMED (N, N, N^1, N^1-tetraethylene-diamine) ammonium pensulfate	45
	TOTAL	$1,295
2.	sodium barbitol	200
	anti-serum	1,500
	starch	100
	Whatman #3 filter and Reeves Angel papers	120
	Tris buffer	60
	glassware	100
	pipets-disposable, nondisposable micro, 5 ml, 10 ml	200
	freezer boxes for sample storage for serum and slides	200
	TOTAL	$2,480
3.	BAPNA	50
	trypsin	120
	TOTAL	$170
4.	Radial Immunodiffusion Material	400
	TOTAL	$400

GRAND TOTAL $4,345

10035942

16. Other sources of financial support:
List financial support from all sources, including own institution, for this and related research projects.

CURRENTLY ACTIVE

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates
NONE			

PENDING OR PLANNED

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates
Please see letter accompanying this application.			

letter states "We plan tentatively to apply for other sources of funding but have not done so as yet. If you wish I would be glad to send copies of future requests to you."

It is understood that the investigator and institutional officers in applying for a grant have read and accept the Council's "Statement of Policy Containing Conditions and Terms Under Which Project Grants Are Made."

Principal investigator

Typed Name Hugh E. Evans, M.D.

Signature Hugh E. Evans M.D. Date 6/25/75

Telephone 212 (Area Code) 240-1000 (Number) 1776 (Extension)

Checks payable to

Jewish Hospital and Medical Center of Brooklyn

Mailing address for checks

555 Prospect Place

Brooklyn, New York 11238

Responsible officer of institution

Typed Name Mr. Philip C. Abrams

Title Executive Director

Signature Philip C. Abrams Date 6/25/75

Telephone 212 (Area Code) 240-1000 (Number) 1201 (Extension)

10035459 43

THE COUNCIL FOR TOBACCO RESEARCH – U.S.A., INC.

July 11, 1975

Grant application No. 1033

PULMONARY

To: The committee comprising Drs. Gardner, Sommers, Jacobson and Wyatt

Subject: Gad Feinstein, Ph.D., Tel Aviv University, Israel
New application No. 1033
"Studies on Peptide Bond Specificities, Active Site and Inhibition of Human Leucocyte Proteases which are Implicated in the Pathogenesis of Pulmonary Emphysema"

History

The applicant took his option of the grant application, though a prior informal inquiry was in the process of being handled as a Case (No. 332).

Request

Application No. 1033 requests $24,683 for the first year of a two year program.

Documents submitted

1. Application dated May 23, 1975 (13 pages, including C.V.s of Drs. Feinstein and Janoff.

2. Five reprints.

D.S.

DS:wg
atts.

1003545945

H1033

THE COUNCIL FOR TOBACCO RESEARCH—U.S.A., INC.
110 EAST 59TH STREET
NEW YORK, N.Y. 10022
(212) 421-8885

Date: May 23, 1975

Application for Research Grant
(Use extra pages as needed)

1. Principal Investigator (give title and degrees): Dr. Gad Feinstein, Senior Lecturer in Biochemistry. B.Sc., M.Sc., Ph.D.

2. Institution & address: Department of Biochemistry, The George S.Wise Center of Life Sciences, Tel-Aviv University, Ramat-Aviv, Tel-Aviv, Israel.

3. Department(s) where research will be done or collaboration provided:
   A. Research will be done at the Biochemistry Department, Tel-Aviv University, Ramat-Aviv, Tel-Aviv, Israel.
   B. Unpaid consultation will be provided by Dr. Aaron Janoff, Dept. of Pathology, SUNY at Stony Brook, Stony Brook, N.Y.

4. Short title of study:
   Studies on Peptide Bond Specificities, Active Site and Inhibition of Human Leucocyte Proteases Which are Implicated in the Pathogenesis of Pulmonary Emphysema

5. Proposed starting date: January 1, 1976

6. Estimated time to complete: December 31, 1977

7. Brief description of specific research aims:
   A. Identification of the amino acid residues that are involved in the binding and catalytic active sites of the two leucocyte proteases, elastase and chymotrypsin-like protease.
   B. Characterization of the peptide bond specificities of these two proteases.
   C. Studies on the degradation of $^{35}SO_4$-labelled tracheobronchial cartilage by purified leucocyte proteases.
   D. Inhibition and inactivation studies of the elastase and chymotrypsin-like protease by naturally occurring inhibitors and synthetic inactivators.

8. Brief statement of working hypothesis:

See attached paper.

9. Details of experimental design and procedures (append extra pages as necessary)

See attached paper.

8. Several lines of investigation have focussed attention on leucocyte proteases, expecially elastase, as mediators of lung destruction in pulmonary emphysema associated with α_1-antitrypsin deficiency (Eriksson [1965]; Mittman [1972]). Levels of leucocyte elastase appear to be related to clinical prognosis in individuals with this disease (Galdston et al. [1973]; Rodriguez et al. [1975]). Elastolytic titer is positively correlated with the severity of lung lesions in animal models of emphysema induced by endotracheal protease instillation (Snider et al. [1974]; Blackwood et al. [1973]). Anatomic emphysema can be produced in dogs by aerosols of human and dog leucocyte homogenates, and the lesion severity is positively correlated with the titer of neutral protease activity in the homogenate (Mass et al. [1972]). Both proteases, leucocyte elastase and chymotrypsin-like protease, were reported (Lieberman and Gawad [1971]) to be capable of degrading human lung tissue. This led to the hypothesis (Lieberman and Gawad [1971]; Lieberman [1973]) that uninhibited proteolytic activities of leucocytes and macrophages result in pathogenesis of pulmonary emphysema.

The purification of human leucocyte elastase was accomplished in this laboratory (Janoff [1973]). More recently, larger scale purification of human leucocyte elastase was reported by us (Feinstein and Janoff [1975a]). The elastase was further characterized with respect to its amino acid composition, molecular weight, synthetic substrate specificity and antigenic properties (including preparation of a monospecific anti-serum). A second human leucocyte protease, chymotrypsin-like neutral protease, was purified by this laboratory (Feinstein and Janoff [1975b]). Some of the physico-chemical properties of the chymotrypsin-like protease were studied including its inhibition by serum protein inhibitors and synthetic inactivators. This latter protease was reported recently, like the granulocyte elastase, to be capable of degrading a natural protein substrate, articular cartilage proteoglycan (Malemud and Janoff [1975]; Feinstein et al. [1975]).

Our working hypothesis is that leucocytic neutral proteases, especially the elastase and the chymotrypsin-like protease, play a significant role in the pathogenesis of pulmonary emphysema. Therefore, proposed studies on peptide-bond specificities, active centers and inhibition of these enzymes could provide important information necessary for their control in individuals with chronic obstructive lung diseases.

List of References for Item No. 8

Blackwood, C.E., Hosannah, Y., Preman, E., Keller, S. and Mandel, I., 1973. Experimental Emphysema in Rats: Elastolytic Titer of Inducing Enzyme as Determinant of the Response, Proc. Soc. Expt. Biol. Med. 144, 450-454.

Erickksson, S. 1965. Studies in α1-Antitrypsin Deficiency. Acta. Med. Scand. 177, Suppl. 432, 1-85.

Feinstein, G. and Janoff, A., 1975a. A Rapid Method for Purification of Human Granulocyte Cationic Neutral Proteases: Purification and Further Characterization of Human Granulocyte Elastase. Biochim. Biophys. Acta., submitted for publication.

Feinstein, G. and Janoff, A., 1975b. A Rapid Method for Purification of Human Granulocyte Cationic Neutral Proteases: Purification and Characterization of Human Granulocyte Chymotrypsin-Like Protease. Biochim. Biophys. Acta., submitted for publication.

Feinstein, G. Malemud, C.J. and Janoff, A., 1975. The Degradation of Articular Cartilage $^{35}SO_4$ by Purified Human Granulocyte Proteases. Manuscript in preparation.

Galdston, M., Janoff, A. and Davis, A.L., 1973. Familial Variation of Leukocyte Lysosomal Protease and Serum α1-Antitrypsin as Determinants in Chronic Obstructive Pulmonary Disease. Am. Rev. Resp. Dis. 107, 718-727.

Janoff, A. 1973., Purification of Human Granulocyte Elastase by Affinity Chromatography. Lab Invest. 29, 458-464.

Lieberman, J. and Gawad, M.A., 1971. Inhibitors and Activators Leukocytic Proteases in Purulent Sputum. Digestion of Human Lung and Inhibition by Alpha$_1$-Antitrypsin. J. Lab. Clin. Med. 77, 713-727.

Lieberman, J., 1973. Involvement of Leukocytic Proteases in Emphysema and Antitrypsin Deficiency. Arch. Environ. Health 27, 196-200.

Malemud, C.J. and Janoff, A., 1975. Identification of Neutral Proteases in Human Neutrophile Granulocytes which Degrade Articular Cartilage Proteoglycans. Arthritis and Rheum., in press.

Mass, B., Ikeda, T., Meranze, D.R., Weinbaum, G. and Kimbel, P., 1972. Induction of Experimental Emphysema. Cellular and Species Specificity. Am. Rev. Resp. Dis. 106, 384-391.

Mittman, C., Ed. "Pulmonary Emphysema and Proteolysis". Academic Press. New York and London. 1972.

Rodriguez, J.R., Seals, J.E., Radin, A., Lin, J.S., Mandel, I. and Turino, G.M., 1975. The Role of Leucocyte Lysosomal Elastase in the Pathogenesis of Obstructive Lung Disease. Clin. Res. 23, 349A.

Snider, G.L., Hayes, J.A., Franzblau, C., Kagan, H.M., Stone, P.S. and Korthy, A.L., 1974. Relationship Between Elastolytic Activity and Experimental Emphysema-Inducing Properties of Papain Preparations. Am. Rev. Respt. Dis. 110, 254-262.

9. Planned Experimental Procedures

A. Identification of the amino acid residues that are involved in the binding and catalytic active sites of the two proteases, elastase and chymotrypsin-like protease. Purified enzymes will be labeled by use of radioactive-labeled specific active-site directed reagents. The labeled enzymes will be degraded by proteolytic enzymes, trypsin and subtilisin, into peptides which will be separated from each other by various procedures like ion-exchanges chromatography, molecular sieving and electrophoresis. The amino acid sequencing of the purified radioactively labeled peptides will give the sequence of amino acids in the active sites of the leucocyte proteases.

B. Characterization of the peptide-bond specificities of these two proteases. Proteins with well known amino acid sequences will be subjected to proteolysis by the purified leucocyte proteases. The peptides obtained will be separated from each other. From the amino acid composition of the peptides it will become evident which peptide bonds in proteins are susceptible to hydrolysis by the leucocyte proteases. In addition a series of small peptides will be tested for their susceptibility to hydrolysis by the proteases. The elucidation of substrate specificity of the enzymes will help to design synthetic inactivators of the proteases.

C. Studies on the degradation of $^{35}SO_4$-labeled tracheobronchial cartilage by purified leucocyte proteases. Lung $^{35}SO_4$-labeled cartilage will be prepared by injection of $Na_2{}^{35}SO_4$ to rabbits which will be sacrificed after 18-24 hours. Tracheal cartilage rings and bronchial cartilage plates will be removed, cleaned and used as substrate for the action of the proteases. The release of soluble $^{35}SO_4$ from the lung tissue will indicate that proteolysis has taken place. The kinetics of the proteolysis and its inhibition by various inhibitors will be studied.

D. Inhibition and inactivation studies. These will include the testing of the capacities of naturally occurring inhibitors from a variety of sources to inhibit the leucocyte proteases. Synthetic inactivators will also be tested for their capacities to specifically inactivate these proteases. This approach will be followed with the purpose of developing inhibitors or inactivators with potential prophylactic application in subclinical stages of the disease.

10. Space and facilities available (when elsewhere than item 2 indicates, state location): All the departmental facilities of the the department of biochemistry at Tel-Aviv University will be available for this research. These include: Analytical Ultracentrifuge, Amino Acid Analyzer, High Resolution Spectrophotometers (Cary 14 etc.), High Voltage Electrophoresis, Preparative Ultracentrifuges, Potentiometric Titrator, Spectroflourometer, Gas Liquid Chromatography, etc.

of radioactive-labeled specific active-site directed reagents. The labeled enzymes will be degraded by proteolytic enzymes, trypsin and subtilisin, into peptides which will be separated from each other by various procedures like ion-exchanger chromatography, molecular sieving and electrophoresis. The amino acid sequencing of the purified radioactively labeled peptides will give the sequence of amino acids in the active sites of the leucocyte proteases.

B. Characterization of the peptide-bond specificities of these two proteases. Proteins which have known amino acid sequences will be [illegible] peptides obtained will be separated from each other and the amino acid composition of the peptides it will be [illegible]

11. Additional facilities required: Recorder, U.V. monitor and High pressure pump.

C. Studies on the degradation of ^{35}S-labeled [illegible] cartilage [illegible] prepared by injection of $Na_2{}^{35}SO_4$ to rabbits which will be sacrificed [illegible] proteolysis and its inhibition by various inhibitors will be studied.

D. [illegible]

12. Biographical sketches of investigator(s) and other professional personnel (append):

13. Publications: (five most recent and pertinent of investigator(s); append list, and provide reprints if available).

14. First year budget:

A. Salaries (give names or state "to be recruited")	% time	Amount
Professional (give % time of investigator(s) even if no salary requested)		
Dr. Gad Feinstein	33	REDACTED
Technical		
Technician (To be recruited)	100	REDACTED
	Sub-Total for A	REDACTED

B. Consumable supplies (by major categories)

Chemical Reagents	- 2500
Ion Exchangers	- 1000
Blood	- 1000
Enzymes and Proteins	- 1000
Glassware	- 1500
Animals	- 1000
	Sub-Total for B REDACTED

C. Other expenses (itemize)

Manuscript Typing and Reprints	- 250
Indexing Service	- 200
Books	- 150
Domestic Travel in Israel	- 300
	Sub-Total for C 900
	Running Total of A + B + C 17,985.-

D. Permanent equipment (itemize)

Recorder	- 1400
UV Monitor	- 1500
High Pressure Pump	- 1300
	Sub-Total for D 4,200

E. Indirect costs (15% of A+B+C) E 2,698.-

Total request 24,883.-

15. Estimated future requirements:

	Salaries	Consumable Suppl.	Other Expenses	Permanent Equip.	Indirect Costs	Total
Year 2	R	9,000	1,000	--	2,700	20,700
Year 3	--	--	--	--	--	--

10035459S2

16. Other sources of financial support:
List financial support from all sources, including own institution, for this and related research projects.

CURRENTLY ACTIVE

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates
Protein Inhibitors in Synovial Fluids of Patients with Joint Diseases	Israeli Ministry of Health	$4000	October 1, 1975

PENDING OR PLANNED

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates

It is understood that the investigator and institutional officers in applying for a grant have read and accept the Council's "Statement of Policy Containing Conditions and Terms Under Which Project Grants Are Made."

Checks payable to

Mailing address for checks

Principal investigator

Typed Name Dr. Gad Feinstein

Signature Gad Feinst Date May 23, 1975

Telephone Israel 03 416-111 749
Area Code Number Extension

Responsible officer of institution

Typed Name I. Levanon

Title Director Research Authority

Signature I. Levanon Date

Telephone Area Code Number Extension

100354953

CURRICULUM VITA

GAD FEINSTEIN, Ph.D.

Personal Data: Born: REDACTED Married: REDACTED

Citizenship: REDACTED Children: REDACTED

Education:

REDACTED Studies. The Hebrew University of Jerusalem, Israel. B.Sc. (1962) in Agriculture.

REDACTED Studies. The University of California, Davis. M.Sc. (1963) in Food Science.
Thesis: Chemical and Physical Properties of the Proteolytic Enzymes of Stem Bromelain.
Instructor - Prof. John R. Whitaker..

Ph.D. (1966) in Comparative Biochemistry.
Thesis: Chemistry of the Inhibitors of Proteolytic Enzymes and Studies of the Mechanism of Action.
Instructor - Prof. Robert E. Feeney.

REDACTED Post-graduate studies. Biology Department, Brookhaven National Laboratory, Upton, New York, with Dr. Elliot N. Shaw.
Subject: Enzyme Chemistry.

Experience:

1962-1966	Graduate Research Assistant, Department of Food Science, University of California, Davis, California.
1966-1968	Research Associate, Biology Department, Brookhaven National Laboratory, Upton, New York.
1968-1973	Lecturer, Biochemistry Department, Tel-Aviv University, Tel-Aviv, Israel.
1973-	Senior Lecturer in Biochemistry (Tenure).
1974-1975	On a Sabbatical Leave from Tel-Aviv University.
	Visiting Lecturer in Pathology, Pathology Department, Basic Health Sciences, State University of New York at Stony Brook, Stony Brook, New York.

No. 13 Recent and Pertinent List of Publications

1. Gad Feinstein, Abraham Kupfer and Mordechai Sokolovsky.
N-Acetyl-(L-Ala)$_3$-p-Nitroanilide as a New Chromogenic Substrate for Elastase.
Biochem. Biophys. Res. Commun. 50, 1020-1026 (1973).

2. Gad Shtacher, Rachel Maayan and Gad Feinstein.
Proteinase Inhibitors in Human Synovial Fluid.
Biochem. Biophys. Acta 303, 138-147 (1973).

3. Emmanual Shapira, Nili Peylan-Ramu, Yoav Ben-Yoseph, Gad Feinstein and Mordechai Sokolovsky.
Specific Immunoassay for Quantitive Determination of Human Chymotrypsin in Intestinal Content.
Israel J. Med. Sci. 10, 1086-1091 (1974).

4. Gad Feinstein, Ronny Hoffstein, Joseph Koifman and Mordechai Sokolovsky.
Human Pancreatic Proteolytic Enzymes and Protein Inhibitors: Isolation and Molecular Properties.
Europ. J. Biochem. 43, 569-581 (1974).

5. Gad Feinstein, Ronny Hoffstein and Mordechai Sokolovsky.
Isolation of Human Pancreatic Inhibitor and Study of Its Interaction with Mammalian and Human Proteases.
Proteinase Inhibitors, Bayer-Symposium V, p. 199-212 (H. Fritz, H. Tschesche, L.J. Greene and E. Truscheit, Eds.). Springer-Verlag, Berlin. Heidelberg.New York. 1974.

6. Gad Feinstein and Aaron Janoff.
A Rapid Method for Purification of Human Granulocyte Cationic Neutral Proteases: Purification and Characterization of Human Granulocyte Chymotrypsin-Like Protease.
Biochim. Biophys. Acta., submitted for publication.

7. Gad Feinstein and Aaron Janoff.
A Rapid Method for Purification of Human Granulocyte Cationic Neutral Proteases: Purification and Further Characterization of Human Granulocyte Elastase.
Biochim. Biophys. Acta., submitted for publication.

8. Gad Feinstein, Charles J. Malemud and Aaron Janoff.
The Degradation of Articular Cartilage $^{35}SO_4$ by Purified Human Granulocyte Proteases.
Manuscript in preparation.

CURRICULUM VITAE (Date of preparation: 5/73)

AARON JANOFF

Born: REDACTED REDACTED

PROFESSIONAL APPOINTMENTS

February 1, 1971: Professor of Pathology, State University of New York at Stony Brook
1966-January 1971: Associate Professor, Department of Pathology, N.Y.U. School of Medicine
1961-1966 : Assistant Professor, N.Y.U. School of Medicine, Department of Pathology
1959-1961 : Instructor, N.Y.U. School of Medicine, Department of Pathology
1958-1959 : Research Associate, N.Y.U. School of Medicine, Department of Pathology
1953-1958 : Teaching Fellow, New York University, Department of Biology

ADMINISTRATIVE APPOINTMENTS - N.Y.U. SCHOOL OF MEDICINE

Executive Admissions Committee
Pass/Fail Grading System Committee
Graduate Advisor, Basic Science Program, Department of Pathology
Medical Student Advisor to First Year Class

ADMINISTRATIVE APPOINTMENTS - S.U.N.Y., STONY BROOK

Director, Graduate Program in Experimental Pathology
Graduate Studies Committee, Graduate Council, Admissions Committee (Medici
SOCIETY MEMBERSHIPS Appointments, Promotions and Tenure Standing Committe

REDACTED

REDACTED

HONORS, AWARDS

Phi Beta Kappa
A.B., cum laude
NSF Predoctoral Fellow, 1956-1958
USPHS Career Development Awardee, 1962-1971

TEACHING EXPERIENCE - N.Y.U. SCHOOL OF MEDICINE

"General Pathology" (Section: Inflammation and Infectious Disease)
"Subcellular and Molecular Pathology" (Section: Lysosomes)
"Biology of Cancer" (Section: Proteases and Co-Carcinogenesis)
Pathology Graduate Students Journal Club (Preceptor)

-Health Sciences Center, SUNY Stony Brook

General Pathology for Medical Students
General Pathology for Nurses, Physicians Associates and Allied Health Professionals
Lysosomes; Structure and Function of Biomembranes; Carcinogenesis (Grad. Cours

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INVITED SPEAKER - INTERNATIONAL MEETINGS

Vth International Congress on Angiology, Paris, France, 1964
Vth International Symposium on Immunopathology, Punta Ala, Italy, 19
Symposium on Pulmonary Emphysema and Proteolysis, City of Hope National Medical Center, Pasadena, California, 1971
IInd International Symposium on the Biochemistry of the Acute Aller Reactions, Brook Lodge, Michigan, 1971
Symposium on Neutrophil Proteases as Mediators of Tissue Injury (Chairman), Atlantic City, New Jersey, 1972
Gordon Conference on Lysosomes, Andover, New Hampshire, 1972
The New York Academy of Sciences (Biochemistry Section), 1972
NY Academy of Sciences Symposium on Rheumatoid Arthritis (Session Chairman) 1974
Cold Spring Harbor Symposium on Proteases and Biological Growth Control 1974

GOVERNMENT SERVICE

Pulmonary Diseases Advisory Committee (National Heart & Lung Institute) 1974-1977

RESEARCH INTERESTS

Purification of Human Leukocyte Proteases; Role of Leukocyte Proteases in the Mediation of Pulmonary Emphysema and Rheumatoid Arthritis; Synthetic Inhibitors of Leukocyte Proteases; Bacterial Degrading Functions of Human Leukocyte Protease

List of References (Dr. A. Janoff) for Item No. 13

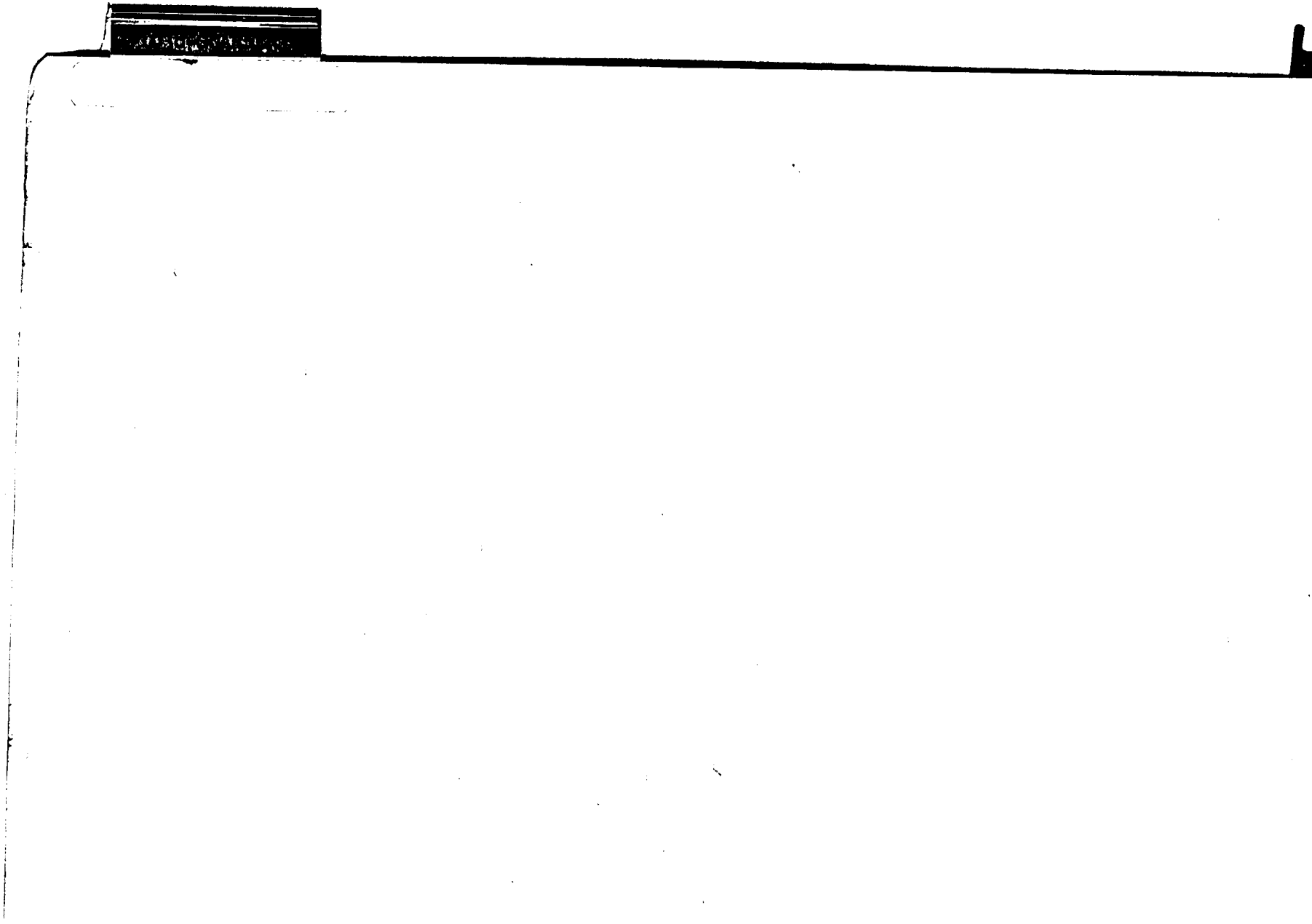
1) Janoff, A. Human Granulocyte Elastase: Further Delineation of its Role in Connective Tissue Damage. Am. J. Path. 68: 579, 1972.

2) Janoff, A. Neutrophil Proteases in Inflammation. Am. Rev. Med. 23: 177, 1972.

3) Galdston, M., Janoff, A. and Davies, A. Levels of Leukocyte Lysosomal Elastase and Serum Alpha 1-Antitrypsin as Determinants in the Expression of Chronic Obstructive Pulmonary Disease. Am. Rev. Resp. Dis. 107: 718, 1973.

4) Janoff, A. Purification of Human Granulocyte Elastase by Affinity Chromatography. Lab. Invest. 29:458, 1973.

5) Janoff, A., Blondin, J. Sandhaus, R.A., Mosser, A. and Malemud, C. Human Neutrophil Elastase: In Vitro Effects on Natural Substrates Suggest Important Physiological and Pathological Actions in: Cold Spring Harbor Symposium on Proteases and Biological Growth Control (eds: E. Reich, D. Rifkin and E. Shaw) Cold Spring Harbor Press, Cold Spring Harbor, NY, in press.



THE COUNCIL FOR TOBACCO RESEARCH – U.S.A., INC.

July 18, 1975

Grant Application No. 1038

PULMONARY

To: The committee comprising Drs. Bing, Gardner and Jacobson

Subject: Joseph J. Guarneri, Ph.D., Long Island Jewish-Hillside Medical Center, Long Island, New York
New application No. 1038
"Influence of Cigarette Smoke on the Response of the Alveolar Macrophage System to Inhaled Bacteria"

History

CTR support of this investigator goes back to 1960. In March 1974 the SAB denied a continuation application on the basis that funding was not justified until a significant amount of his previously collected data was published. However, an extension to March 31, 1975 without additional funds was granted. In March 1975 the SAB denied a further application (No. 547E) partly on the above basis, and partly because the proposal was much too broad and included areas which appeared to be outside the expertise of the applicant.

The studies in the present application appear to be concerned with those areas which the applicant can be reasonably expected to fulfill.

In the past year, Guarneri has submitted two full manuscripts to CTR for review, has published several abstracts and one full paper: one paper is in preparation (see pages 14 - 15of grant application).

Request

Application No. 1038 requests $27,897 for the first year of a three year plan: estimates for the second and third years are: $29,534 and $31,362, respectively.

Documents Submitted

1. Application dated June 30, 1975 (29 pages including CV^{s} of Drs. Guarneri and Shidlovsky).

2. Three manuscripts (Nos. 22/25/28)

3. Addendum I -- Background Material and Supporting Data.

4. Addendum II -- Pertinent Personal Publications (3 pages).

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Comment

1. Selection of the type of cigarette to be used in these studies (University of Kentucky Reference Cigarettes) will be decided by CTR staff.

2. Papers and abstracts listed in Addendum II will be forwarded on request.

David Stone

DS/LP
Encls.

1038

THE COUNCIL FOR TOBACCO RESEARCH–U.S.A., INC.
110 EAST 59TH STREET
NEW YORK, N. Y. 10022
(212) 421-8865

Application for Research Grant
(Use extra pages as needed)

Date: 6/30/75

1. Principal investigator (give title and degrees):
Joseph J. Guarneri, Ph.D., Attending Microbiologist
Long Island Jewish-Hillside Medical Center/Queens Hospital Center
Affiliation

2. Institution & address:
Long Island Jewish-Hillside
Medical Center
New Hyde Park, N.Y. 11040

Research Performance Site & Address
Long Island Jewish-Hillside Medical
Center/Queens Hospital Center Affil.
82-68 164 St., Jamaica, N.Y. 11432

3. Department(s) where research will be done or collaboration provided:
Division of Microbiology, Department of Laboratories

4. Short title of study: Influence of Cigarette Smoke on the Response of the Alveolar Macrophage System to Inhaled Bacteria.

5. Proposed starting date: January 1, 1976

6. Estimated time to complete: 3 years

7. Brief description of specific research aims: The proposed project emanates from observations made in this and other laboratories which demonstrate the following: (a) during in vivo smoke-exposure or incubation in bronchopulmonary fluid obtained from the lungs of smoke-exposed animals, alveolar macrophages do not effectively kill bacteria and (b) in contrast, alveolar macrophages from long term cigarette smokers and smoke-exposed animals rapidly ingest and destroy bacteria when challenged with bacteria in the absence of cigarette smoke or smoke-products. Although important, these observations fail to provide a scientific basis for explaining the impairment of macrophage function by smoke and the subsequent recovery of alveolar macrophages from the adverse effects of in vivo smoke-exposure. To this end, methods and protocols are presented to distinguish between a direct effect of cigarette smoke on the phagocytic properties of alveolar macrophages and those related to a suppression of phagocytosis-promoting factors present in the lung. To accomplish these objectives, the following determinants of effective macrophage function, as a lung phagocyte, will be studied in the absence of cigarette smoke, during in vitro and in vivo smoke-exposure and after the cessation of smoke inhalation: (a) chemotactic responsiveness to bacterial stimuli, (b) phagocytosis including the adherence of bacteria to macrophage surfaces and the rate at which membrane-associated bacteria are ingested, (c) the rate of intracellular destruction of ingested bacteria and (d) the availability and immunologic competence of phagocytosis-promoting factors present in serum and the lungs.

8. Brief statement of working hypothesis:

Epidemiologic and clinical reports suggests an increased incidence of respiratory infections among smokers, especially heavy smokers. In support of such a relationship, experimental studies demonstrate that acute and chronic exposure to cigarette smoke impairs the clearance of inhaled bacteria from the lungs of animals. The alveolar macrophage has been implicated as the most important intrapulmonary defense mechanisms which protects the host against bronchopulmonary damage by inhaled infectious and toxic agents. Repeated human and animal studies indicate that cigarette smoke increases rather than depletes the available supply of alveolar macrophages in the lung and does not impair cell viability or the mobilization of macrophages in response to the inhalation of live bacteria. In addition, alveolar macrophages obtained from smokers and smoke-exposed animals retain their ability to effectively kill bacteria, in vitro, in the absence of smoke. In contrast, it has been shown that during in vitro and in vivo exposure to cigarette smoke the ability of alveolar macrophages to destroy bacteria is compromised. These data suggest a direct effect of smoke on the phagocytic and/or bactericidal properties of macrophages. In support of this view, recent studies performed in this laboratory under test conditions that dissociate phagocytosis from bacterial destruction demonstrate that in vitro smoke-exposure has an adverse effect on the rate at which bacteria bound to macrophage membranes are ingested and destroyed. These findings must be confirmed under in vivo conditions of smoke-exposure in the absence of possible artifacts. For this reason a significant part of the proposed research is devoted to an evaluation of the effects of in vivo smoke-exposure on phagocytic and bactericidal activity studied as sequential and independent processes. The data obtained from other studies also suggest that impairment of macrophage function by smoke inhalation may be mediated by a smoke-occasioned augmentation of the supply and/or immunologic integrity of phagocytosis-promoting factors present in the lung. In support of this view are two fundamental observations made in this laboratory: (1) in the absence of normal serum or immunologically active components of the lung obtained by bronchopulmonary lavage, alveolar macrophages do not kill bacteria, in vitro, and (2) bacterial challenge in bronchopulmonary lavage material obtained from smoke-exposed animals results in a marked suppression of the antibacterial properties of alveolar macrophages. These findings indicate the need for a direct investigation of the immunological accompaniments that add to the functional activity of alveolar macrophages in the absence of smoke, during smoke-exposure and after the cessation of smoke inhalation. Finally, the clinical significance of the data obtained from the proposed investigation is strengthened by the fact that the alveolar macrophage and immune systems in the respiratory tract of animals appear to respond to cigarette smoke in a manner similar to that of man.

See pages 16 to 36 for a summary of the Background Material and Supporting Data that serve as a basis for the studies proposed in this grant.

9. Details of experimental design and procedures (append extra pages as necessary). See pages 2A-1 to 2A-13.

Table of Contents

9. Experimental Design: Study of the Influence of Cigarette Smoke on the Phagocytic Properties of Alveolar Macrophages.

Reference Response System: Effective Phagocytosis and Destruction of *Staphylococcus aureus* in an *In Vitro* Phagocytosis System

Conditions of Smoke Exposure:

- *In Vitro*: Normal Alveolar Macrophages Exposed to Cigarette Smoke in Tissue Culture Flasks
- *In Vivo*: Alveolar Macrophages Harvested from the Lungs of Rabbits Exposed to Cigarette Smoke

Test Conditions of Bacterial Challenge:

- Tissue Culture Media plus Normal and Immune Rabbit Serum
- Tissue Culture Media plus the Immunologic Components in the Lung Retrieved from the Lungs of Normal and Immune Rabbits by Bronchopulmonary Lavage

Parameters of Phagocytosis to be studied:

1. Chemotactic Responsiveness to Bacterial Stimuli
2. Attachment of Bacteria to Macrophage Surfaces
3. Ingestion of Membrane Associated Bacteria
4. Intracellular Destruction of Ingested Bacteria by Macrophages

The Concentration and Activity of Mediators of Phagocytosis in Serum and Lung to be Assayed:

Serum	Lung
1. Complement (C'3, C'5)	1. Complement (C'3, C'5)
2. IgG	2. IgG
	3. SIgA

Experimental Design

A. Quantitative Measurements of the Influence of Cigarette Smoke on Alveolar Macrophage Activity: There is sufficient evidence to suggest that the alveolar macrophage is critically important in pulmonary defense against inhaled bacteria. The specific components of alveolar macrophage activity, as a lung phagocyte, which enable it to serve this key role in pulmonary defense have been demonstrated. Alveolar macrophages are mobilized following the inhalation of bacteria and can effectively ingest and destroy bacteria deposited in the lungs. Accordingly, studies are presented to assess the effect of cigarette smoke on the chemotactic and phagocytic properties of alveolar macrophages. The proposed studies were designed to assess the individual and comparative effects of in vivo and in vitro smoke-exposure on the antibacterial activity of alveolar macrophages, and the phagocytosis-promoting properties of serum and the immunologic components of the lung retrievable by bronchopulmonary lavage. To accomplish these objectives, all studies will be performed both under in vitro and in vivo conditions of smoke-exposure and will include alveolar macrophages from non-immunized rabbits challenged with Staphylococcus aureus in tissue culture media individually supplemented with one of the following additions: (a) normal rabbit serum, (b) serum from rabbits immunized against S. aureus, (c) bronchopulmonary lavage from normal rabbits and (d) bronchopulmonary lavage from rabbits immunized against S. aureus. This basic approach is consistent with the specific aim of the grant proposal to distinguish between smoke-associated changes in the antibacterial activity of alveolar macrophages due to a direct effect of cigarette smoke on the alveolar macrophage proper and those ascribable to a smoke-induced augmentation of the immunological accompaniments necessary for effective antibacterial action by alveolar macrophages. This information is also needed to provide a scientific basis for explaining the impairment of macrophage function by cigarette smoke and the rapid recovery of alveolar macrophages from the adverse effects of smoke inhalation.

The conditions of in vivo and in vitro exposure to cigarette smoke, in vitro bacterial challenge and methods for harvesting alveolar macrophages, collecting the immunologic components of the lung by bronchopulmonary lavage and immunization schedules are presented in detail under Methods of Procedure on pages 2A-8 to 2A-11.

(1) Studies of the Chemotactic Responsiveness of Alveolar Macrophages: An understanding of the influence of cigarette smoke on chemotactic responsiveness is desirable because the rapid mobilization and migration of alveolar macrophages to pulmonary sites following the inhalation of infectious and toxic agents determines the prevention and limitation of bronchopulmonary damage from these materials. The data obtained from these studies will be correlated with observation made in this laboratory (1,2) which demonstrate that cigarette smoke induced a selective mobilization of alveolar macrophages and did not interfere with the mobilization of alveolar macrophages provoked by the inhalation of viable S. aureus

Since the chemotactic response to a given stimulus is the adjusted composite of changes in cell adhesiveness and motility, both properties of the alveolar macrophage will be investigated. The experimental system used by Carruthers (3) to study leucocyte motility will be adapted to alveolar macrophages. This method is based on the ability of motile cells to move through the pores of a membrane filter. Two O-ring joints separated by a millipore filter are clamped together and sealed to form 2 distinct chambers. Fixed numbers of alveolar macrophages ($1-3 \times 10^7$) suspended in tissue culture media will be introduced into chamber number one which is then sealed with a paraffin plug. The second chamber will be filled with 5 mg. of insoluble potato starch. After an initial period of incubation at 37°C to permit monolayer formation, the chambers will be inverted so that the test cells will then be on the bottom side of the filter, and the chemotactic substance, if present, is on the top side of the filter. The chamber will then be placed in an incubator at 37°C for 4 hours. At the end of various hourly intervals, the filter will be removed, stained with hematoxylin and subjected to microscopic study. In this way, the number of cells on both sides of the filter will be enumerated and used as an index of the migratory response elicited by stimulatory agents. Separate studies are planned to evaluate the changes in cell adhesiveness that occur in phagocytizing alveolar macrophages. The method of studying the adhesive properties of blood leukocytes reported by Allison and Lancaster (4) will be adapted to alveolar macrophages. Test tube cultures of fixed numbers of lung phagocytes suspended in tissue culture media will be challenged with known numbers of S. aureus. Under conditions favoring maximum phagocytosis, microscopic methods will be used to determine the formation of cell aggregates by phagocytizing macrophages. Changes in cell adhesivenss will be evaluated under the same experimental conditions described above to assess motility.

(2) Studies of Phagocytosis and Bacterial Killing: Previous investigations have established the importance of phagocytosis and bacterial destruction by alveolar macrophages in the initial defense of the lung against bacterial invasion. Phagocytosis and bacterial killing are sequential events that must be measured simultaneously to accurately determine the overall antibacterial properties of a phagocytic cell. At present, it is also possible to dissociate phagocytosis from bacterial destruction so that each process can be investigated independently. Failure to utilize methods that permit quantitative determinations of overall antibacterial activity, phagocytosis and intracellular inactivation of phagocytized bacteria can and have lead to misleading reports.

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(a) Simultaneous Measurements of Phagocytosis and Bacterial Destruction: Fixed numbers of S. aureus are added to known numbers

of alveolar macrophages adhering to the flat surface of a tissue culture flask containing tissue culture media. Immediately after bacterial challenge and at various intervals thereafter, the extracellular fraction containing free or unphagocytized bacteria is isolated by removing the supernatant fluid from macrophage cultures with a pipette. To recover the viable bacteria within macrophages (macrophage fraction), the tissue culture flasks are vigorously shaken after adding glass beads and the bacteria-laden macrophages are lysed by treatment with cold distilled water. In all studies the protocol included control flasks containing the tissue culture medium inoculated with S. aureus. Bacterial counts are obtained from the extracellular fraction, macrophage fraction and control flasks by a standard pour plate technique. The rate of disappearance of viable organisms from the intracellular fraction is used to express the percent bacteria phagocytized, while the difference between the total viable bacterial population in control flasks and the numbers of bacteria in the extracellular and macrophage fractions is used to determine the percent bacteria killed by alveolar macrophages.

(b) Phagocytosis: Since phagocytosis has been shown to be a two stage event consisting of the adherence to and subsequent destruction of ingested bacteria by phagocytes, methods will also be used to differentiate bacterial attachment from ingestion. After challenging macrophages cultures with S. aureus for 1.5 hrs, the macrophage fraction is separated from the extracellular fraction, as previously described, and incubated for 15 min in 3.0 ml of tissue culture medium containing 2.5% trypsin (Grand Island Biological Company). The bacteria released from the surface of macrophages by trypsin (adherence fraction) are recovered by decanting the supernatant and washing the macrophages three times with 2.5 ml of Hanks' solution. The bacteria associated with the trypsin-treated macrophages are present within macrophages and may be referred to as the ingested fraction of the total bacteria recovered. To recover the ingested fraction of bacteria, the trypsin-treated macrophages will be lysed by treatment with cold sterile distilled water. The numbers of viable bacteria present in the adherence and ingested fractions are determined from bacterial counts obtained from nutrient agar pour plates incubated at 37°C for 48 hrs. Under these conditions, it is possible to determine the relative number of viable bacteria attached to and within alveolar macrophages (ingested) at various times during the phagocytic event: Ratio of the no. viable bacteria in the adherence fraction to the no. viable bacteria in the ingested fraction.

(c) Bacterial Inactivation - Intracellular Destruction of Ingested Bacteria: In other studies, intracellular destruction of ingested S. aureus by alveolar macrophages will be studied in the absence of all other aspects of the phagocytic event. For this purpose

lysostaphin (Schwartz Mann) will be used (final concentration of 150 ug/ml) to kill all extracellular bacteria as well as those adhering to macrophage membranes. Lysostaphin is a muralytic enzyme which does not enter phagocytes and selectively eliminates extracellular staphylococci (5). Macrophage cultures are challenged with S. aureus for 1 hr at 37°C. At this time, 0.1 ml of lysostaphin is added to all macrophage cultures. After 30 minutes incubation at 37°C, duplicate flasks will be removed from the incubator and treated with 0.3 ml of trypsin to inactivate and neutralize the lysostaphin. Viable intracellular bacteria will be released by agitation with glass beads and osmolysis with cold sterile distilled water. Two hours later, duplicate flasks will be taken out of the incubator as outlined above. Viable bacterial counts will be made 48 hrs later and the % intracellular killing of S. aureus is determined as follows:

$$\text{Intracellular killing} = 100\% - \frac{\text{No. viable bacteria in macrophage cultures 2.0 hrs after bacterial challenge period}}{\text{No. viable bacteria in macrophage cultures immediately after bacterial challenge period}} \times 100$$

THE ABOVE METHODS ARE CURRENTLY IN USE IN THIS LABORATORY

B. Quantitative Measurements of the Influence of Cigarette Smoke on Phagocytosis-Promoting Factors in Serum and Extractable from the Lung by Bronchopulmonary Lavage: There is evidence to suggest that cigarette smoking alters the relative distribution of the immunoglobulins and complement proteins in serum, tracheobronchial mucosa and lung (6,7). The C'3 and C'5 complement proteins and the immunoglobulin IgG and SIgA are of specific interest for the following reasons: (a) C'3 and C'5 are the principle complement components responsible for the chemotactic responsiveness of phagocytes to bacterial agents (8), (b) C'3 and IgG are the best characterized opsonins found in serum and bronchial fluid. In this regard, IgG has been shown to increase the rate at which bacteria attach to receptor sites on the surface of phagocytes, and IgG and C'3 enhance the rate of bacterial ingestion by phagocytes (9) and (c) SIgA is the major secretory immunoglobulin produced locally in tracheobronchial mucosa. Because of its ability to alter bacterial growth rates (10), bacterial viability (11) and the adherence of bacteria to mucosal surfaces (12), SIgA may be a central factor in the role of the alveolar macrophage as a lung phagocyte. For these reasons studies are proposed to assess the effect of cigarette smoke on the concentration and activity of the above humoral factors in serum and bronchial fluid. This information is needed to differentiate between smoke-occasioned changes in macrophage function due to a direct effect of cigarette smoke on the alveolar macrophage and those ascribable to an augmentation of the immunological accompaniments necessary for effective antibacterial action by alveolar macrophages.

(1) Studies of the Influence of Cigarette Smoke on the Concentration of Immunoglobulins and Complement Proteins Extractable from the Lung by Bronchopulmonary Lavage: The relative distribution of C'3, C'5, IgG and SIgA will be determined in normal rabbits and rabbits immunized against S. aureus prior to and after exposure to cigarette smoke for 1, 5, 10 and 15 days. Quantitation of the humoral factors in question will be made by electroimmunodiffusion as outlined under Materials and Methods of Procedure on page 2A-10.

(2) Studies of the Influence of Cigarette Smoke on the Phagocytosis-Promoting Activity of the Immunoglobulins IgG and SIgA and Complement Components C'3 and C'5: To accomplish the above, experiments will be performed to establish the individual and relative contributions of C'3, C'5, IgG and SIgA to the antibacterial activity of alveolar macrophages. For this purpose, separate studies will be performed to compare the ability of alveolar macrophages to phagocytize and kill S. aureus in serum and bronchopulmonary lavage fluid prior to and after treatment of the serum and bronchial fluid with individual inhibitors and/or absorbants of C'3, C'5, IgG and SIgA. The SIgA determinations apply only to the bronchopulmonary lavage studies. In the course of these studies, direct measurements will also be made of the activity (immunologic competence) of each of the immunoglobulins and complement components by equivalence point titrations against their respective antisera. The selective inhibition and immunoglobulin-complement activity studies will be performed with alveolar macrophages harvested from non-immune animals and challenged with S. aureus under the following conditions of bacterial challenge: (a) in Hanks' solution containing serum from either normal rabbits or rabbits immunized against S. aureus prior to and after in vitro exposure of the sera to various concentrations of whole cigarette smoke and (b) in bronchopulmonary fluid obtained from either normal rabbits or rabbits immunized against S. aureus not exposed to cigarette smoke, immediately after in vivo exposure to cigarette smoke and after the termination of experimental smoke inhalation.

The methods used to achieve selective depletion and/or absorption of each humoral component and technique used to perform equivalence point titrations are presented in detail on pages 2A-11.

Materials and Methods of Procedure

A. Conditions of In Vivo Smoke-Exposure: A continuous stream of puffed cigarette smoke generated from commercial non-filtered 70 mm cigarettes is produced by an automatic smoke machine* designed to sequentially puff 30 cigarettes to a 20 to 23 mm butt length. The smoke generating apparatus is adjusted to deliver a 35 ml puff of 2 sec duration from each cigarette once every min. Cigarette smoke is drawn through a smoke-exposure chamber at a rate of 25 liter per min by a secondary airflow of room air created by a vacuum pump located at the downstream end of the exposure chamber. Airflow is monitored with a rotometer and controlled to produce chamber concentrations of cigarette smoke of approximately 1 part of whole cigarette smoke and 25 to 30 parts room air. Control animals are placed in a chamber and sham-smoked with unlighted cigarettes and subjected to a secondary airflow of room air.

In the present studies Albino New Zealand male rabbits will be exposed daily for 1.0 hr to whole cigarette smoke for 1, 5, 10 and 15 days over a 3 week period. These are the same conditions of smoke-exposure used in this laboratory to assess the influence of acute and extended exposure to cigarette smoke on the clearance of inhaled bacteria from the respiratory tract (see Addendum I, Supporting Data, pages 19 to 33.

B. Conditions of In Vitro Smoke-Exposure: A commercial brand of non-filtered cigarette is attached to a 30 ml syringe by a rubber tube and smoke is produced by withdrawing the barrel of the syringe at a rate of approximately 18 ml per second. Six successive "puffs" of cigarette smoke are introduced into the syringe and emptied by removing a rubber tube containing the lighted cigarette. The smoke from the seventh puff is introduced into the tissue culture flasks with a sterile hypodermic needle. The protocol of each study included a control flask (bacteria only) and macrophage cultures not exposed to cigarette smoke and control and corresponding macrophage preparations exposed to cigarette smoke. Under these conditions, macrophage cultures are exposed for 1.5 hours to whole cigarette smoke.

C. Conditions of In Vitro Bacterial Challenge: Alveolar macrophages harvested from rabbits are placed in tissue culture flasks containing a tissue culture medium (Hanks' solution) and normal serum, immune serum, normal bronchopulmonary lavage, immune bronchopulmonary in accordance with the protocol of each study. Immediately after inoculation with fixed numbers of Staphylococcus aureus (FDA 209P, phage type 42D) and at 15 min intervals over a 90 min period after bacterial challenge, the macrophage cultures are processed to measure overall antibacterial activity, phagocytosis and bacterial destruction as outlined on pages 24-25. To determine if the effects of in vivo smoke-exposure

*Progressive Engineering Co., Richmond, Va.

on macrophage function are reversible, overall antibacterial activity, phagocytosis and bacterial destruction will be measured immediately following exposure to cigarette smoke for 1, 5, 10 and 15 days and 1, 5 and 10 days after the termination of experimental smoke inhalation.

D. Harvesting of Alveolar Macrophages: Alveolar macrophages are harvested from the lungs of albino rabbits weighing 1.0 to 2.0 kilograms by the general method of Myrvik et al(13). The animals are killed by injecting air in the marginal ear vein. This method of sacrificing animals is used to avoid any depressant effects that anesthetics may have on alveolar macrophage activity. The trachea is exposed and canulated with a sterile polyethylene tube, and 17 ml of sterile Hanks' solution is introduced in the intact lung. It is allowed to remain in the lung for 6 min, and then is recovered by aspiration. The lavage fluid is centrifuged at 2000 rpm for 10 min. The resultant supernatant fluid is separated from the cell pellet by decanting thus dividing the aspirated material into an alveolar macrophage fraction and an acellular fluid fraction containing the immunologic contents of the lung retrievable by bronchopulmonary lavage. The acellular fluid fraction is stored at - 70°C and later processed as described below (see E). The numbers of macrophages present in the macrophage fraction are enumerated in a bright line hemocytometer and differential counts are made on Wright stained smears. By this method 95% of the macrophages harvested are viable as determined by the Eosin Y dye exclusion test (14).

E. Processing of Bronchopulmonary Lavage to Characterize Immunologic Components Extractable from the Lung: The acellular fraction of lung harvests (see above D) will be concentrated 5, 10 and 50 fold by ultrafiltration in Diaflow Membranes (Amicon Corp.). To date this method of processing the acellular fraction of the lung has resulted in the recovery of bronchopulmonary lavage material capable of promoting the phagocytic activity of alveolar macrophages in the absence of exogenous serum.

F. Immunization Schedule:

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(1) Parenteral Immunization: Antiserum specific to S. aureus will be produced in rabbits as presented by Oeding (15). A suspension of 2 to 4 x 10^9 staphylococci/ml is centrifuged at 6,000 rpm for 20 min and the resultant supernatant is discarded. The pellet containing staphylococci is resuspended in 4% formalized saline and stored at 4°C for 24 hrs. At this time, the formalin-treated bacteria are streaked out on blood agar to test for the presence of viable staphylococci. If bacterial growth is detected, the formalin suspension of bacteria is stored at 4°C for 24 hr intervals until total bacterial death is achieved. The suspension is then washed and centrifuged 3 times and resuspended in saline. The rabbits are treated with the formalin killed bacteria according to the following schedule. Rabbits are injected intravenously (iv)

on 3 successive days with 0.1 ml, 0.2 ml and 0.4 ml of the formalized S. aureus. After 5 days the rabbits are now injected iv with 0.4 ml, 0.6 ml and 0.8 ml of formalin-killed staphylococci on 3 successive days. Five days later the rabbits receives iv injections with 0.8 ml, 1.0 ml and 1.0 ml of formalin-treated S. aureus on three successive days. Finally, five days after the termination of the last set of intravenous inoculations with formalin-killed bacteria, the animals are bled and serum reactivity is tested by double immunodiffusion against S. aureus. Upon confirmation of the achievement of a high titer, all the animals will be exsanguinated by cardiac puncture. In addition, at this time, lungs of the sacrificed rabbits will be washed out by bronchopulmonary lavage, concentrated and tested for activity against S. aureus as outlined above

(2) Intranasal Immunization: Since it has been shown that the intranasal or aerogenic route of immunization favors the production of SIgA agglutinative antibody on bronchial secretions in response to a given antigen (10) this method will be used for the purpose of studying the influence of SIgA on the phagocytic capacity of alveolar macrophages. Rabbits will be immunized with heat-killed S. aureus (10^9 organisms/ml) by intranasal inoculation utilizing the procedure and immunization schedule of Reynolds et al (15). In short, 2 ml of S. aureus suspension will be instilled intranasally with an eye dropper 3 times per week for 2 weeks or until agglutinating antibodies are detected in bronchial lavage material.

G. Quantitation of C'3, C'5, IgG and SIgA: Quantitation of the immunoglobulins and complement proteins in serum will be performed by the method of electroimmunodiffusion as outlined by Merrill et al (17). The antirabbit IgG, C'3 and C'5 sera and the appropriate antigen standards needed for the procedure will be purchased from Microbiological Associates. The same methodology will be used to quantitate C'3, C'5 and IgG in bronchopulmonary lavage material. In addition anti SIgA and SIgA standards previously prepared in this laboratory by the method of Cebra and Robins (18) will be used to quantitate the amount of SIgA extractable from the lungs by bronchopulmonary lavage.

With regards to the electroimmunodiffusion method proper, glass slides (1" x 3") are coated with 0.3% Ionagar containing 0.05% glycerol. The slides are then coated with 2 ml of Agarose in a vernol buffer (pH 8.6, 0.05 M) containing multiple dilutions of antiserum against the specific antigen under study. After the slides solidify, 3 circular wells are cut into the agar along the short axis of each slide and filled with 4 ul of the test serum or bronchial fluid. The slides are now subjected to electrophoresis for 90 min at 150 volts. After electrophoresis the slides are washed in saline and distilled water for 1 hr and finally dried at 37°C. The dried slides

are stained with 0.1% Amido Schwartz in 1.0% acetic acid. The length of the resultant precipitin lines formed by the migration of the antigens present in serum or bronchial lavage is measured and plotted against the precipitin lines formed by standard antigens of known concentration. A new standard curve will be constructed for each humoral component studied.

H. Selective Depletion/Absorption of C'3, C'5, IgG and SIgA: Depletion of C'3 will be attained by reacting serum or bronchial lavage material with Zymosan (1.35 mg/ml) at 37°C for 1 hr (18). The test serum or bronchopulmonary lavage material is centrifuged and the supernatant free of C'3 is recovered.

The absorption of C'5, IgG and SIgA will be performed by modifications of a standard immunoadsorption technique described in detail in published reports (20,21). Sepharose 4B is swelled, equilibrated in an appropriate buffer and coupled with either anti C'5, IgG or SIgA sera at 4°C on a rotary shaker overnight. The antibody-gel complex is placed in a beaker containing serum or bronchopulmonary lavage material and mixed gently for 2 hrs at room temperature. The suspension is centrifuged at 2,500 rpm for 20 min at 4°C to pellet the Sepharose beads containing the antigen-antibody complex. The supernatant free of C'5, IgG or SIgA is decanted and filter-sterilized.

The completeness of depletion or absorption of C'3, C'5, IgG and SIgA achieved by the methods presented above will be monitored by double immunodiffusion against appropriate antisera. Failure of serum and bronchopulmonary lavage material to react under conditions of Ouchterlony analysis will be used as an index of the completeness of depletion or absorption. Since the reaction between IgG and anti IgG is complement dependent, serum and bronchopulmonary lavage levels of complement will also be measured.

I. Equivalence Point Titrations: Equivalence points will be determined by the method described by Campbell et al (22). Serial dilutions are made of antisera to C'3, C'5, IgG and SIgA and a fixed amount of serum or bronchopulmonary lavage material will be added to each antisera dilution. The highest antisera dilution showing the greatest amount of precipitation is considered the zone of equivalence. The time of the reaction is also noted.

References to Experimental Design and Materials and Methods of Procedure.

1. Guarneri, J. J. and Laurenzi, G. A.: Influence of cigarette smoke on pulmonary defense against inhaled bacteria. Bact. Proceedings, 1972 (Abstract No. M 170).

2. Guarneri, J.: Influence of in-vivo exposure to whole cigarette smoke on the antibacterial properties of alveolar macrophages. Abstracts of the Annual Meeting of the American Society for Microbiology, 1975 (Abstract No. B43).

3. Carruters, R.M.I.: Method of studying normal variation effect of physical alterations in environment, and effect of iodacetate. Canad. J. Physiol. and Pharm. 44:457, 1966.

4. Allison, Jr., F. and Lancaster, M.G.: Studies on the factors which influence the adhesiveness of Leukocytes in vitro. Ann. of N. Y. Acad. Science 115:936, 1964.

5. Tan, J.S., Watanakunakorn, C. and Phair, J.P.: A modified assay of neutrophil function: Use of lysostaphin to differentiate defective phagocytosis from impaired intracellular killing. J. Lab. Clin. Med., 78:318, 1971.

6. Lewis, D.M., Lapp, N.L. and Burrell, R.: Quantitation of secretory immunoglobulin A in chronic pulmonary disease. Am. Rev. Resp. Dis. 101:55, 1970.

7. Reynolds, H.Y. and Newball, H.H.: Analysis of proteins and respiratory cells obtained from human lungs by bronchial lavage. J. Lab. Clin. Med. 84:559, 1974.

8. Stossel, T.P.: Phagocytosis. New Eng. J. Med., 290:717, 1974.

9. Schultz, D.R.: The Complement System. Monographs in Allergy Volume 6. S. Karger Co., Basel, 1971.

10. Reynolds, H.Y. and Thompson, R.E.: Pulmonary host defenses: I. Analysis of proteins and lipids in bronchial secretions and antibody responses after vaccination with Pseudomonas aeruginosa. J. Immunol. 111:358, 1973.

11. Burdon, D.W.: Bactericidal action of immunoglobulin A. Proceeding Path. Soc. Great Britain and Ireland. 5:VII, 1972.

12. Williams, R.C. and Gibbons, R.J.: Inhibition of bacterial adherence by secretory immunoglobulin A: A mechanism of antigen disposal. Science. 177:697, 1973.

13. Myrvik, Q.N., Leake, E.S. and Fariss, R.: Studies on pulmonary macrophages from the normal rabbit: A technique to procure them in a high state of purity, J. Immunol. 86:128, 1961.

14. Hanks, J. H. and Wallace, J. H.: Determination of cell viability. Proc. Soc. Exp. Biol. Med. 98:188, 1958.

15. Oeding, P.: Agglutinability of pyrogenic staphylococci at various conditions. Acta Path. Microbiol. Scand. 41:310, 1957.

16. Reynolds, H. Y., Thompson, R. E. and Devlin, H. B.: Development of cellular and humoral immunity in the respiratory tract of rabbits to pseudomonas lipopolysaccharide. J. Clin. Invest. 53: 1351, 1974.

17. Merrill, D., Hartley, T. F. and Claman, H. N.: Electroimmunodiffusion. J. Lab. Clin. Med. 69:151, 1967.

18. Cebra, J. and Robbins, J.: Gamma A immunoglobulin from rabbit colostrum. J. Immunol. 92: 12, 1966.

19. Weir, D.M.: Handbook of Experimental Immunochemistry. Blackwell Scientific Publications, Oxford, 1973.

20. Frommel, D., Dupuy, J.M. and Litman, G.W., et al.: Use of immunoadsorption technique in the preparation of chemical agammaglobulinemia. J. Immunol. 105: 1292, 1970.

21. CNBr - Activated Sepharose 4B for Immobilization of Biopolymers. Pharmacia Fine Chemicals, Inc., Piscataway, N. J., 1973.

22. Campbell, D. H., Garvey, J. S., Cremer, N. E. and Susdorf, D. H. Methods in Immunology. 2nd ed. W. A. Benjamin, Inc., New York, 1970.

10. Space and facilities available (when elsewhere than item 2 indicates, state location):

The Microbiology Research Laboratories are located as a complex in the Triboro Hospital at Queens Hospital Center, Jamaica, New York. They include: (a) an aerosol exposure laboratory, (b) a smoke-exposure laboratory, (c) individual laboratories for macrophage studies and microbiology, (d) 2 rooms for storage space and refrigerators. Animal quarters are provided in another area of the hospital. The entire laboratory area occupies approximately 700 square feet.

The equipment in this area includes a complete bacterial aerosol generating and exposure system with mixing chambers and decontaminating units, and an Anderson apparatus for measuring particle size of bacterial aerosols: A cigarette smoke generating apparatus and exposure chamber and a sequential sampler and gas liquid chromatography unit for determining concentration of the particulate and gas phase of cigarette smoke. Other major instrumentation present include the following: (a) standard microscopes, (b) 1 infusion pump, (c) a centrifuge, (d) 2 large refrigerators, (e) sonic dismembranator, (f) 2 water baths and shaker, (g) 1 freezer, (h) 2 incubators and 1 environmental chamber, (i) 4 vacuum pumps, (j) pH meter, (k) Beckman DU2 recording spectrophotometer, (l) spectronic 20 spectrophotometer, (m) Gilson respirometer, (n) a lyophilizer unit, (o) immuno and disc electrophoresis apparatus, (p) large autoclave, (σ) analytical balance, (r) flash evaporator and (s) Isco automatic fraction collector for liquid chromatography. There is also equipment for qualitative and quantitative bacteriology studies, tissue homogenation, administration of gas mixtures and animal surgery. A Revco deep freezer (-75°C) is also available. High performance scintillation counters capable of isotope work are present in the hospital and available for research use. In addition, a fully equipped laboratory capable of performing histological and electron microscopy studies are available to the Division of Microbiology.

11. Additional facilities required:

None.

12. Biographical sketches of investigator(s) and other professional personnel (append):

See pages 6 - 13.

13. Publications: (five most recent and pertinent of investigator(s); append list, and provide reprints if available).

See pages 14 - 15 and Addendum II.

14. First year budget:

A. Salaries (give names or state "to be recruited") Professional (give % time of investigator(s) even if no salary requested)	% time	Amount
Joseph J. Guarneri, Ph.D. Principal Investigator	20 hrs/60 hrs	REDAC
Boris A. Shidlovsky, Ph.D.	7 hrs	REDACTED
Technical		
Research Associate (to be appointed)	35 hrs/100%	$15,600
	Fringe Benefits	2,808
	Sub-Total for A	18,408

B. Consumable supplies (by major categories)

See page 4A for detailed list

Sub-Total for B	4,850

C. Other expenses (itemize)

Journals and Books	200
Travel	800
Sub-Total for C	1,000
Running Total of A + B + C	24,258

D. Permanent equipment (itemize)

None

Sub-Total for D	-
E	3,369
Total request	27,897

E. Indirect costs (15% of A+B+C)

15. Estimated future requirements:

	Salaries	Consumable Suppl.	Other Expenses	Permanent Equip.	Indirect Costs	Total
Year 2	19,881	4,800	1,000	-	4,002	29,534
Year 3	21,471	4,800	1,000	-	4,090	31,362

B. Consumable Supplies

1.	Cigarettes for smoke studies	$1,000
2.	Mice	100
3.	Rabbits	2,000
4.	Immunodiffusion Plates, Antisera, Radial Immunodiffusion kit and templates, Antigen standards	600
5.	Tissue culture glassware, tissue culture media and bacteriology media, and nebulizers	550
6.	Petri dishes and plastic disposable pipets and bacteriologic filters	600
		$4,850

16. Other sources of financial support:

List financial support from all sources, including own institution, for this and related research projects.

CURRENTLY ACTIVE

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates
The Influence of Extended Exposure to Cigarette Smoke on Pulmonary Resistance to Infection as Related to Alveolar Macrophages and Mucociliary Function.	The Council for Tobacco Research, U.S.A. Grant Nos. 547C, 547 CR-1 and 547 CR-2	$65,152	7/1/71 to 6/30/75
Important Determinants of Pulmonary Resistance to Infection, Alcoholic Intoxications.	Long Island Jewish-Hillside Medical Center Grant No. 274	33,600	5/3/74 to 12/31/75

PENDING OR PLANNED

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates
None	Renewal of LIJ/Hillside Medical Center Grant No. 274	25,000	1/1/76 - 12/30/76

It is understood that the investigator and institutional officers in applying for a grant have read and accept the Council's "Statement of Policy Containing Conditions and Terms Under Which Project Grants Are Made."

Principal investigator

Typed Name Joseph J. Guarneri, Ph.D.

Signature [redacted] Date 7/16/75

Telephone [redacted]
Area Code Number Extension

Checks payable to

Long Island Jewish-Hillside Medical Center

Mailing address for checks

Mrs. Eva Meyer, Grant Manager
Long Island Jewish-Hillside Medical Center
New Hyde Park, New York 11040

Responsible officer of institution

Typed Name James E. Mulvihill, D.M.D.

Title Vice President
Research and Education

Signature James E. Mulvihill D.M.D. Date 7-16-75

Telephone [redacted] 2111
Area Code Number Extension

12 Biographical Sketch Joseph J. Guarneri, Ph.D. Principal Investigator

NAME: Joseph J. Guarneri, Ph.D.

ADDRESS: HOME: REDACTED

OFFICE: Long Island Jewish-Hillside Medical Center/Queens Hospital Center
82-68 164th St., Jamaica, New York 11432

TELEPHONE: HOME: REDACTED
OFFICE: 212-990-2335

BIRTHDATE AND PLACE: REDACTED

CITIZENSHIP: REDACTED

MARITAL STATUS: REDACTED

SOCIAL SECURITY #: REDACTED

EDUCATION: REDACTED New York University; B.A. (Biology), 6/49
- Saint John's University; M.S. (Microbiology), 6/63
REDACTED - Saint John's University; Ph.D. (Microbiology), 6/66

INTERNSHIP RESIDENCY: Not applicable.

MILITARY SERVICE: 1/49 - 3/46 - Sergeant, U.S. Army; Medical Corp., Camp Lee, Virginia

BOARD STATUS: American Society for Microbiology; Certification as Specialist in Public Health and Medical Laboratory Microbiology.
American Academy of Microbiology; Fellowship (Pending).

TYPE OF PRACTICE: Not applicable. 1003545981

LICENSURE STATUS: Certificate of Qualification for Director of a Clinical Microbiology Laboratory, City of New York, Department of Health.

ACADEMIC POSITIONS:

9/61 - 6/66 - Research Associate, Division of Respiratory Diseases, New Jersey College of Medicine and Dentistry.

6/66 - 6/68 - Instructor in Medicine, Department of Medicine, New Jersey College of Medicine and Dentistry, Jersey City, New Jersey.

7/68 - 1/72 Director, Pulmonary Aerobiology Research Laboratory, Division of Infectious Diseases, Department of Medicine, Saint Vincent Hospital Worcester, Mass.

ACADEMIC POSITIONS:

1/72	Attending Microbiologist, Long Island Jewish-Hillside Medical Center/Queens Hospital Center.
6/73	Associate Clinical Professor Pathology, SUNY at Stony Brook.
6/73	Coordinator Allied Health Sciences, Queens Hospital Center, Jamaica, New York.
9/73	Associate in Microbiology, St. John's University, Jamaica, New York.

MEMBERSHIP IN PROFESSIONAL SOCIETIES:

REDACTED

REDACTED

REDACTED

REDACTED

HONORS AND AWARDS:

Sigma XI, Saint John's University, 1963

3/68 to 5/69 - Public Health Service. H.I.H. Award #A.I. 08963-01. Title: The Mechanism of Pulmonary Resistance to Infection. Principal Investigators: G.A. Laurenzi, M.D. Associate Director: J.J. Guarneri, Ph.D. - Amount $67,769.

7/68 to 6/69 - The Council for Tobacco Research-U.S.A. Grant Award #547. Title: The Effect of Cigarette Smoke on the Nature and Function of Alveolar Macrophages. Principal Investigator: G.A. Laurenzi, M.D. Co-Investigator: J. J. Guarneri, Ph.D. - Amount $36,135.

9/68 to 6/71 - Saint Vincent Hospital Research Foundation. Title: The Role of the Alveolar Macrophage in Pulmonary Defense Against Inhaled Bacteria. Principal Investigator: J. J. Guarneri, Ph.D. Amount: $ 18,667.

6/69 to 5/72 - Public Health Service. H.I.A. Award #AI 08963-02. Title: The Mechanism of Pulmonary Defense Against Infection. Principal Investigator: G.A. Laurenzi, M.D. Associate Director: J.J. Guarneri, Ph.D. - Amount: $128,137.

1003545982

HONORS AND AWARDS:

7/69 - 6/71 - The Council for Tobacco Research - U.S.A. Grant Award #547BR1. Title: The Effect of Cigarette Smoke on the Immunological and Metabolic Function of Alveolar Macrophages. Principal Investigator: J.J. Guarneri Ph.D. - Amount: $29,380.

7/71 - 6/72 - Saint Vincent Hospital Research Foundation. Title: The Influence of Bacterial Species on the Anti-bacterial Activity of Alveolar Macrophages. Principal Investigator: J. J. Guarneri, Ph.D. Amount $ 4,000.

7/71 - 6/75 - The Council for Tobacco Research - U.S.A. Grant Award # 547C. Title: The Influence of Extended Exposure to Cigarette Smoke on Pulmonary Resistance to Infection as Related to Alveolar Macrophage and Muco-ciliary Function. Principal Investigator: J.J. Guarneri, Ph.D. - Amount: $63,000.

5/74 - 6/75 - Long Island Jewish-Hillside Medical Center #274. Title: Important Determinants of Pulmonary Resistance to Infection, Alcoholic Intoxication. Principal Investigator: J. J. Guarneri, Ph.D. - Amount: $22,413.

PUBLICATIONS: (Papers published or in press).

1. Laurenzi, G.A., Guarneri, J.J., Endriga, R.B. and Carey, J.P.: Clearance of Bacteria by the Lower Respiratory Tract. Science: 142: 1572-1573, 1963.

2. Laurenzi, G.A., Guarneri, J.J. and Endriga, R.B. "Important Determinants in Pulmonary Resistance to Bacterial Infections." In the Pathogenisis of Chronic Obstructive Broncho-Pulmonary Disease. In Mitchell R.S.: Progress in Research in Emphysema and Chronic Bronchitis, New York, S. Karger, 1965, p. 48-59.

3. Laurenzi, G.A., Guarneri, J.J. and Endriga, R.B.: Important Determinants in Pulmonary Resistance to Bacterial Infection. Medicinia Thoracalis 22: 48-59, 1965.

4. Laurenzi, G.A., and Guarneri, J.J.: A Study of the Mechanisms of Pulmonary Resistance to Infection: The Relationship of Bacterial Clearance to Ciliary and Alveolar Macrophage Function. Symposium on Structure, Function, and Measurement of Respiratory Cilia. Am. Rev. of Respiratory Dis. 93: 134-141, 1966.

5. Laurenzi, G.A., Yin, S., Collins, R., Guarneri, J.J.: Mucus Flow in the Mammalian Trachea. Current Research in Chronic Obstructive Lung Disease. U.S. Public Health Service Publication No. 1787: 27-40, 1967.

6. Laurenzi, G.A., Yin, S., and Guarneri, J.J.: The Adverse Effect of Oxygen on Tracheal Mucus Flow. New England J. of Med. 279: 333-339, 1968.

7. Guarneri, J.J. and Laurenzi, G.A.: The Effect of Alcohol on the Mobilization of Alveolar Macrophages. J. of Lab. and Clinical Med. 72: 40-51, 1968.

8. Combs, T.J., Guarneri, J.J., and Pisano, M.A. The Effect of Sodium Chloride on the Lipid Contents and Fatty Acid Composition of Candida Albicans. Mycologia LX: 1232-1239, 1968.

9. Guarneri, J.J.: Clearance of Inhaled Bacteria from the Murine Respiratory Tract. In Developments In Industrial Microbiology. American Institute of Biological Sciences, Washington, D.C., Volume 16, 1974

10. Khan, F., Guarneri, J.J. and Sierra, M.F.: Primary Pulmonary Sporotrichosis Complicated by Perirectal. Am. Rev. Respiratory Dis., 1975

11. Guarneri, J.J.: Influence of Acute Exposure to Cigarette Smoke on the Alveolar Macrophage System. Submitted to J. of Lab. and Clin. Med.

ABSTRACTS: (abstracts published and presented or read by title) **1003545984**

1a. Laurenzi, G.A., Guarneri, J.J. and Endriga, R.B.: Bacterial Clearance from the Lung of Mice, Fed. Proc.: 22: 255, 1963.

2a. Laurenzi, G.A., Endriga, R.B., Guarneri, J.J. and Carey, J.P.: Important Determinants in Resistance to Pulmonary Infection. J. Clinical Invest. 949: 42, 1963.

3. Laurenzi, G.A., Guarneri, J.J. and Endriga, R.B.: Important Determinants in Pulmonary Resistance to Infection: Proceeding of Seventh Aspen Conference on Bronchitis and Pulmonary Emphysema. p. I-4, 1964.

4a. Laurenzi, G.A., Collins, B.J., Yin S. and Guarneri, J.J.: The Adverse Effects of High Oxygen Breathing and Hypoxia. J. Clin. Invest. 45: 1035, 1966.

5a. Laurenzi, G.A., Yin, S., Collins, B.J. and Guarneri, J.J.: Mucus Flow in the Mammalian Trachea. Proceeding of the Tenth Aspen Conference. p. 100, 1966.

6a. Laurenzi, G.A., Collins, B.J., Yin, S. and Guarneri, J.J.: Adverse Effect of High Oxygen Breathing on Tracheobronchial Mucus Flow. Amer. Rev. Resp. Dis. Vol. 96: 152, 1967.

7a. Guarneri, J.J., Combs, T.J. and Pisano, M.A.: Lipid Components of Candida Stellatoide Bacterial Proc. p. 84, 1967.

8a. Guarneri, J.J.: Lipid Composition of Candida Stellatoides. Dissertation Abstracts 27: 3614-B, 1967.

9a. Guarneri, J.J. and Laurenzi, G.A.: The Mobilization of Alveolar Macrophages as a Pulmonary Defense Mechanism Against Inhaled Bacteria. Bacterial Proc. p. 100, 1968.

10a. Laurenzi, G.A., Yin, S., Collins, B.J. and Guarneri, J.J.: Mucus Flow in the Mammalian Trachea. Public Health Service Publication No. 1787, p. 27, 1967.

11a. Combs, T.J., Guarneri, J.J. and Pisano, M.A.: Effect of Growth Conditions on the Fatty Acid Composition of Candida Albicans. The Third Symposium on Yeasts, Delft-Hague, The Netherlands, June 2 - 7, 1969.

12a. Guarneri, J.J. and Laurenzi, G.A.: The Effect of Cigarette Smoke on Alveolar Macrophage Numbers. The 148th Annual Meeting of the New York City Branch of the American Society for Microbiology, New York, N.Y., Feb. 25, 1971.

13a. Guarneri, J.J. and Laurenzi, G.A.: Influence of Cigarette Smoke on Pulmonary Defense Against Inhaled Bacteria. Alveolar Macrophage Numbers and Viability, The 72nd Annual Meeting of the American Society for Microbiology, Philadelphia, Pa., May 26, 1972.

14a. Guarneri, J.J. and Sierra, M.F.: Antibacterial Activity of Alveolar Macrophages Against Staphylococcus aureus. The 197th Annual Meeting of The American Society for Microbiology, New York City Branch, Wagner College, Staten Island, New York.

15a. Guarneri, J.J.: Influence of In vitro Exposure to Cigarette Smoke on the Antibacterial Properties of Alveolar Macrophages. The 197th Annual Meeting of The American Society for Microbiology, New York City Branch, Wagner College, Staten Island, New York, April 15, 1974.

16a. Guarneri, J.J.: Influence of Extended Exposure to Cigarette Smoke on Pulmonary Defense against Inhaled Bacteria. 74th Annual Meeting of The American Society for Microbiology, Chicago, Ill., May 12- 17, 1974.

17a. Guarneri, J.J.: Clearance of Inhaled Bacteria from Murine Respiratory Tract. 25th Annual Meeting of the Society for Industrial Microbiology, Memphis, Tenn., Aug. 11 - 16, 1974. (Abstract)

18a. Guarneri, J.J.: Influence of Acute Exposure to Cigarette Smoke on Pulmonary Defense Mechanisms. 14th Interscience Conference on Antimicrobial Agents and Chemotherapy, San Francisco, Calif. Sept. 11 - 13, 1974. (Abstract).

19a. Guarneri, J.J.: Influence of In Vivo Exposure to Whole Cigarette Smoke on the Antibacterial Properties of Alveolar Macrophages. 1975 Annual Meeting of American Society for Microbiology.

20a. Guarneri, J.J., Goldstein, J. and Shidlovsky, R.A.: Effect of In Vitro Exposure to Cigarette Smoke on the Antibacterial Properties of Alveolar Macrophages. 1975 Annual Meeting of American Society for Microbiology.

21a. Guarneri, J.J., Laurenzi, G.A., and Sierra, M.F.: Influence of Trichlorofluoromethane and Dichlorodifluoromethane on the Clearance of Bacteria from the Lungs of Mice. 26th Annual Meeting of the Society for Industrial Microbiology, Kingston, R.I., August 17th - 22nd, 1975.

PRESENTATIONS: (papers given by invitation and thesis)

1p. Guarneri, J.J.: The Inhibition of Bacteria by Aconitic Acid. Master's Thesis, St. John's University, June 9, 1963.

2p. Laurenzi, G.A., Guarneri, J.J. and Endriga, R.B.: Important Determinants in Pulmonary Resistance to Infection: Processing of Seventh Aspen Conference on Bronchitis and Pulmonary Emphysema, p. 1 - 4, 1964.

3p. Laurenzi, G.A., Yin, S., Collins, B.J. and Guarneri, J.J.: Mucus Flow in the Mammalian Trachea. Proceeding of the Tenth Aspen Conference, p. 100, 1966.

4p. Guarneri, J.J.: Lipid Composition of Candida Stellatoidea. Ph.D. Thesis, St. John's University, June 12, 1966.

Biographical Sketch - Boris A. Shidlovsky, Ph.D.

NAME: Boris A. Shidlovsky, Ph.D.

REDACTED

ADDRESS: Home: -
Office: Long Island Jewish-Hillside Medical Center/
Queens Hospital Center
82-68 164 Street, Jamaica, New York 11432

TELEPHONE: Home: REDACTED
Office: 212-990-2337

DATE OF BIRTH:

MARITAL STATUS: REDACTED

EDUCATION:

R - New York University B.A.
- St. John's University M.S.
- St. John's University Ph.D.

MILITARY SERVICE: 1942 - 1946 U.S. Army Medical Corps. senior non-commissioned officer.

LICENSURE STATUS: Certificate of Qualification for Director of a Clinical Microbiology Laboratory, City of New York, Department of Health.

PROFESSIONAL EXPERIENCE:

1951-1961	Div. of Surg. Res. Lab. Harlem Hospital, New York City, Bacteriologist-in-charge.
1961-1962	Microbiology Department, New York University Dental School, New York, Research Associate.
1962-1963	Misericordia Hospital, New York, Chief Bacteriologist
1963-1966	Morrisania Hospital (Montefiore-Morrisania Hospital Affil.), Chief Bacteriologist
1966-1969	Quinton Research Labs/Merck & Co., Inc. (Senior Research Microbiologist)
1969-1974	Associate Professor at Monmouth College, West Long Branch, New Jersey
1974	Assistant Attending Microbiologist, Long Island Jewish-Hillside Medical Center/ Queens Hospital Center.

MEMBERSHIPS IN PROFESSIONAL SOCIETIES:

REDACTED

REDACTED

10035459R7

MEMBERSHIPS IN PROFESSIONAL SOCIETIES:

New York City Branch-Am. Soc. Microbiol.
Society of General Microbiology
Society of Industrial Microbiology
SIGMA Xi Club of St. John's University
Theobald Smith Society

RESEARCH INTERESTS: Antimicrobial agents and host defense mechanisms

13. Publications Pertinent to Material Covered in Grant Proposal*

1. Guarneri, J.J. and Laurenzi, G.A. The Effect of Cigarette Smoke on Alveolar Macrophage Numbers. The 148th Annual Meeting of the New York City Branch of the American Society for Microbiology, New York City, Feb. 25, 1971.

2. Guarneri, J.J. and Laurenzi, G.A. Influence of Cigarette Smoke on Pulmonary Defense Against Inhaled Bacteria: Alveolar Macrophage Numbers and Viability. The 72nd Annual Meeting of the American Society for Microbiology, Philadelphia, Pa., May 26, 1972. (Abstract No. M 170).

3. Guarneri, J.J. and Sierra, M.F. Antibacterial Activity of Alveolar Macrophages Against Staphylococcus aureus. The 1974 Annual Meeting of the American Society for Microbiology, New York City Branch, New York, April 15, 1974.

4. Guarneri, J.J. Influence of In Vitro Exposure to Cigarette Smoke on the Antibacterial Properties of Alveolar Macrophages. The 1974 Annual Meeting of the American Society for Microbiology, New York City Branch, New York, April 15, 1974.

5. Guarneri, J.J. Influence of Extended Exposure to Cigarette Smoke on Pulmonary Defense against Inhaled Bacteria. 74th Annual Meeting of the American Society for Microbiology, Chicago, Ill. May 12 - 17, 1974. (abstract No. M 355).

6. Guarneri, J.J. Clearance of Inhaled Bacteria from the Murine Respiratory Tract. The 25th Annual Meeting of the Society for Industrial Microbiology, Memphis, Tenn., Aug. 11 - 16, 1974.

7. Guarneri, J.J. Influence of Acute Exposure to Cigarette Smoke on Pulmonary Defense Mechanisms. The 14th Interscience Conference on Antimicrobial Agents and Chemotherapy, San Francisco, Calif., Sept. 11-13, 1974. (Abstract No. 181).

8. Guarneri, J. J. Clearance of Inhaled Bacteria from the Murine Respiratory Tract. In: Developments In Industrial Microbiology. American Institute of Biological Sciences, Washington, D.C., Volume 16, 1975.

9. Guarneri, J.J. Influence of Acute Exposure to Cigarette Smoke on the Clearance of Bacteria by the Murine Respiratory Tract. Presented to CTR for Review Prior to Submission to Am. Rev. Resp. Dis.

10. Guarneri, J.J. and Goldstein, J. A study of the In Vitro Interaction Between Alveolar Macrophages and Staphylococcus aureus. The 1975 Annual Meeting of the American Society for Microbiology, New York City Branch, New York. Jan. 14, 1975.

11. Guarneri, J.J. Influence of In Vivo Exposure to Cigarette Smoke on the Antibacterial Properties of Alveolar Macrophages. Abstracts of the Annual Meeting of the American Society for Microbiology, 1975 (Abstract No. B 43).

12. Guarneri, J.J., Goldstein, J. and Shidlovsky, B.: Effect of In Vitro Exposure to Cigarette Smoke on the Antibacterial Properties of Alveolar Macrophages. Abstracts of the Annual Meeting of the American Society for Microbiology, 1975 (Abstract No. B 44).

13. Publications Pertinent to Material Covered in Grant Proposal*

13. Guarneri, J.J. Influence of Acute Exposure to Cigarette Smoke on the Clearance of Bacteria by the Murine Respiratory Tract. Presented to the Council for Tobacco Research Prior to Submission to Am. Rev. Resp. Dis.

14. Guarneri, J.J. Influence of Extended Exposure to Cigarette Smoke on the Clearance of Bacteria by the Murine Respiratory Tract. (In Preparation).

* See Curriculum Vitae page 9 for a complete list of publication.
See Addendum II for copies of publications pertinent to material covered in grant proposal.

THE COUNCIL FOR TOBACCO RESEARCH–U.S.A., INC.

July 3, 1975

Grant Application No. 1041

PULMONARY

TO: The committee comprising Drs. Feldman, Gardner, Sommers and Wyatt

SUBJECT: Joseph M. Lauweryns, M.D., Ph.D., University of Leuven, Belgium
New application No. 1041
"The Neuro-epithelial Bodies: Their Role and Structure as Intrapulmonary Neuro (chemo) Receptors in Normal and Various Physiological, Pharmacological and Pathological Conditions"

History

CTR has supported this distinguished and most productive applicant (Grant No. 741 (renewals and continuations)) since 1970 in studies entitled "The Lymphatics of the Lung. Their Role in Fluid Transport and Clearance of Airborne Particulate Matter in Normal and Experimental Conditions and in Various Lung Diseases". CTR support for these studies terminates December 31, 1975.

Request

Application No. 1041 requests $32,420 for the first year of a three year project. Estimates for the second and third years are $35,662 and $39,229, respectively.

Documents Submitted (attached)

1. Application dated June 16, 1975 (5 pages) with Details of Experimental Design (Addendum I...17 pages), Facilities Available (Addendum II...2 pages).

2. Biographical sketches of Lauweryns, Cokeleaere and Liebens (Appendum III). Only the first page of Lauweryn's CV is attached-- the remaining 26 pages -- this does not include publications -- will be forwarded upon request.

3. List of five most recent and pertinent publications (Addendum IV).

David Stone

DS/lp
Encl.

1041

The Council For Tobacco Research–U.S.A., Inc.

110 EAST 59TH STREET
NEW YORK, N. Y. 10022
(212) 421-8885

Application for Research Grant
(Use extra pages as needed)

Date: June 16th, 1975

1. Principal Investigator (give title and degrees):

– Lauweryns, Joseph-M., M.D., Ph.D., Professor Ordinarius in Microscopic Anatomy and Pathology ; Chairman, Principal Investigator.

2. Institution & address:

Experimental Laboratory of Pulmonary Histopathology, Department of Pathology, University of Leuven, 12, Minderbroedersstraat, B – 3000 LEUVEN – BELGIUM.

3. Department(s) where research will be done or collaboration provided:

Experimental Laboratory of Pulmonary Histopathology, Department of Pathology, University of Leuven, 12, Minderbroedersstraat, B – 3000 LEUVEN – BELGIUM.

4. Short title of study:

The Neuro-epithelial Bodies : their role and structure as intrapulmonary neuro(chemo) receptors in normal and various physiological, pharmacological and pathological conditions.

5. Proposed starting date: January 1, 1976 (first year)

6. Estimated time to complete: From January 1, 1976 till December 31st, 1978.

7. Brief description of specific research aims:

We recently identified throughout the intrapulmonary airways of the human and mammalian lung intramucosal corpuscles or so-called Neuro-epithelial Bodies (NEB's), whose general characteristics have been described in the hereby included pertinent publications. From these earlier observations and preliminary experiments on the effect of hypoxia on these NEB's, we have suggested that the NEB's may provide an intrapulmonary, hypoxia sensitive neuro(chemo)receptor system modulated by the central nervous system in addition to the well-known central and peripheral chemoreceptors. They contain and secrete serotonin and probably also related amines and peptides, which could influence the pulmonary vasoconstrictor response. They could however have various other possible functions, modulating not only pulmonary vasomotion, but also bronchial and bronchiolar mucosal secretion or smooth muscle tone.

During the three-years of this research proposal (1976–1978) we intend to study the pulmonary NEB's along three major lines of investigation : – (1) a further and thorough investigation of their normal morphology, and especially their relationship to the Kultschitzky-like cells and the APUD-series, – (2) an experimental study of their structural reactions under various physiological (hypoxia, hyperoxia, hypoxemia, cross-circulation) and pharmacological (L-DOPA, 5-HTP, nicotine) conditions, – and (3) a light optical study of their incidence and size in lung diseases, mainly associated with hypoxia.

Various techniques which complement each other and are all familiar to us, will be applied : – light optics, histochemistry, morphometry, fluorescence microscopy, microspectrography, transmission electron microscopy and scanning electron microscopy.

Though the lines of investigation are distinct, the study object is identical and the results interrelated. These studies will necessarily end in important and original basic and applied results as regards the structure and functions of the normal and diseased lung. It is obvious that these studies are of immediate and relevant importance in biological tobacco research.

8. Brief statement of working hypothesis:

Having identified the occurrence of NEB's in the human and mammalian lung, it is obviously indicated to apply furthermore the tools of a combined and multidisciplinary investigation to unravel their fine structure and function. This study will moreover benefit of the experience of the same team of investigators who work closely together since several years and who have their personal skill as regards the various techniques to be used.
The NEB's appear as a new area of promising future research and discovery in various basic fields of pulmonary function and structure (e.g. hypoxic pulmonary vasoconstriction).

9. Details of experimental design and procedures (append extra pages as necessary)

See separate pages - Addendum 1

Please note moreover :

During this three-year (1976-1978) research program - outlined in "addendum 1 (details of experimental design and procedures) - we have proposed to study the NEB's along three major lines of investigation, i.e. :

- (1) Further studies on the normal morphology of the NEB's
- (2) An experimental study of the structural reactions of the NEB's under various physiological (hypoxia, hyperoxia, hypoxemia, cross-circulation) and pharmacological (L-DOPA, 5-HTP, nicotine) conditions, and
- (3) A light optical study of the incidence and size of the NEB's in pathological conditions in the human lung.

Though we will start the investigation of the three items at the same time, we will mainly concentrate our efforts during this first year (1976) on item # 2.

10. Space and facilities available (when elsewhere than item 2 indicates, state location):

- All physical facilities are available at our laboratory (as mentioned under item 2), and we will have facilities for scanning electron microscopy either at the university of Ghent or Leuven.
- Separate list of these physical facilities - see addendum 2

11. Additional facilities required:

None

i.e. - Lauweryns, Joseph-M., M.D., Ph.D., principal investigator.
- Cokelaere Marnix, Lic. Biol. Sc., full-time research assistant, doctorandus, Co-investigator.
- Liebens Marc, Lic. Biol. Sc., full-time research assistant, Co-investigator.

12. Biographical sketches of investigator(s) and other professional personnel (append):

See separate pages - Addendum 3

13. Publications: (five most recent and pertinent of investigator(s); append list, and provide reprints if available).

Publications on the NEB's - see Addendum 4
Progress Report of Grant No. 741B, No. 741C - see Addendum 5

14. First year budget:

A. Salaries (give names or state "to be recruited")	% time	Amount
Professional (give % time of investigator(s) even if no salary requested)		
- Lauweryns Joseph	60 %	none
- Cokelaere Marnix	90 %	none
- Liebens Marc	90 %	none
Technical		
- Two laboratory technicians (Kris ARMEE ; Stefaan ONS)	100 %	16.196
Sub-Total for A		16.196

B. Consumable supplies (by major categories)

Animals	3,024
supplies	13.200
Sub-Total for B	16.224

C. Other expenses (itemize)

Sub-Total for C	none
Running Total of A + B + C	32,420

D. Permanent equipment (itemize)

Sub-Total for D	none
E	none
Total request	32.420

E. Indirect costs (15% of A+B+C)

15. Estimated future requirements:

	Salaries	Consumable Suppl.	Other Expenses	Permanent Equip.	Indirect Costs	Total
Year 2	17.816	17.846				35.662
Year 3	19.598	19.631				39.229

16. Other sources of financial support:
List financial support from all sources, including own institution, for this and related research projects.

CURRENTLY ACTIVE

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates
Morphological studies of the lung.	University of Leuven		
	J.M. Lauweryns, Professor, salary	$ 24,000	yearly
	M. Cokelaere, assistant salary	$ 12,000	yearly
	M. Liebens, assistant salary	$ 12,000	yearly
	Salary of 2 technicians	$ 17,400	yearly
	Supplies and animals	$ 7,600	yearly

PENDING OR PLANNED

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates

It is understood that the investigator and institutional officers in applying for a grant have read and accept the Council's "Statement of Policy Containing Conditions and Terms Under Which Project Grants Are Made."

Principal investigator

Typed Name Joseph M. LAUWERYNS

Signature J. Lauweryns Date June 16th, 75

Telephone 016 (Area Code) 22.89.81 (Number) (Extension)

Checks payable to

Prof. P. DE SOMER, Rector of the University

Mailing address for checks

Dr. P. DE SOMER, Rector, Catholic University of Leuven
B - 3000 LEUVEN - BELGIUM

Responsible officer of institution

Typed Name Pieter DE SOMER

Title Rector

Signature [illegible] Date June 20th, 75

Telephone 016 (Area Code) 22.04.31 (Number) 131 or 116 (Extension)

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#1050-TURINO

1003545998

THE COUNCIL FOR TOBACCO RESEARCH–U.S.A., INC.

July 17, 1975

Grant Application No. 1050

PULMONARY

To: The committee comprising Drs. Gardner, Jacobson and Meier

SUBJECT: Gerard M. Turino, M.D., College of Physicians and Surgeons, Columbia University, New York City
New application No. 1050
"Chemical Basis of Tissue Destruction in Obstructive Lung Disease"

History

This application did not go through the "case" procedure as an informal inquiry.

Request

Application No. 1050 requests $105,663 for the first year of a three year project: estimates for the second and third years are $101,140 and $109,230, respectively.

Documents submitted (attached)

1. Application dated July 1, 1975 (22 pages including CVs of Drs. Turino, Fierer, Mandl and Parshley).

2. Addendum 1 (1 page).

Comment

1. Work requiring exposure of animals to NO_2 for prolonged periods will be carried out in collaboration with Dr. G. Freeman, Stanford Research Institute, California.

2. Dr. Mandl (collaborating in this study) has previously received six years of CTR support for studies in this general area (July 1966 through June 1972) in studies entitled "The Role of Hereditary Elastase Inhibitor Deficiency in the Etiology of Pulmonary Emphysema" and "Elastolytic Breakdown in the Etiology of Pulmonary Emphysema".

David Stone

DS/lp
Encls.

1050

THE COUNCIL FOR TOBACCO RESEARCH—U.S.A., INC.

110 EAST 59TH STREET
NEW YORK, N. Y. 10022
(212) 421-8885

RECEIVED
JUL 15 1975

Application for Research Grant
(Use extra pages as needed)

Date July 1, 1975

1. Principal Investigator (give title and degrees):

Gerard M. Turino, M.D.
Professor of Medicine

2. Institution & address:

Columbia University, College of Physicians and Surgeons
630 West 168th Street
New York, New York 10032

3. Department(s) where research will be done or collaboration provided:

Departments of Medicine, Pathology and Obstetrics and Gynecology

4. Short title of study:

Chemical Basis of Tissue Destruction in Obstructive Lung Disease

5. Proposed starting date: October 1, 1975

6. Estimated time to complete: 3 years

7. Brief description of specific research aims:

To define the role of elastase activity of alveolar macrophages and of circulating polymorphonuclear leukocytes in the development of experimental pulmonary emphysema in animals and in the etiology of emphysematous destruction of the lung in human subjects.

1. Within this objective, an attempt will be made to determine differences for alveolar macrophage elastase activity between patients who have obstructive airways disease as compared with normal subjects as has already been demonstrated in this Laboratory for the polymorphonuclear leukocytes between these two groups. In addition other distinctions include differences in elastase activity among patients who have primarily pulmonary emphysema as compared with patients who have primarily asthma or asthmatic bronchitis with less pulmonary parenchymal destruction. Also, distinctions will be sought between normal subjects who have a history of smoking as compared with non-smokers.

2. The response of polymorphonuclear leukocyte and alveolar macrophage elastase activity to the following interventions in experimental animals (rats and hamsters) will be quantified: a) prolonged daily exposure to tobacco smoke over a four to six week period and after cessation, b) prolonged exposure to NO_2 at concentrations in inspired air of 15 ppm and NO_2 and O_3 of .9 ppm each (approximately six weeks), c) pulmonary injury induced by intratracheal and intravenous injection of pancreatic elastase and papain intratracheally.

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7. Brief description of specific research aims (continued)

3. To determine the feasibility of using tissue cultures of alveolar macrophages from normal human lungs and from patients with obstructive lung disease for the identification of levels of elastase activity and to investigate interventions which may either increase or decrease alveolar macrophage elastase activity. These interventions include a) exposure to tobacco smoke (daily exposure for several weeks), b) hypoxia (10 to 15% O_2), c) exposure of cells and media to high CO_2 atmosphere (5 to 10%) in air and d) chemical acidosis of the media induced by addition of low molar concentrations of hydrochloric acid.

8. Brief statement of working hypothesis:

The hypothesis of this study is that destruction of pulmonary parenchyma which is commonly a concomitant of obstructive lung disease in man and is a primary etiological factor in pure forms of pulmonary emphysema in the human population is the result of proteolytic injury to connective tissue components of the lung. Injury to pulmonary elastin is an essential feature of this destructive process and the chemical basis for degradation and disruption of elastin resides in both polymorphonuclear leukocytes which are sequestered in the lung and alveolar macrophages which are normal cellular components of the alveolus. It is hypothesized also that certain environmental factors, such as constituents of tobacco smoke, environmental gases or in vivo tissue factors in the lung, stimulate and perpetuate over-activity of cellular elastase and perhaps other proteases.

9. Details of experimental design and procedures (append extra pages as necessary)

Chemical Basis of Tissue Destruction in Obstructive Lung Disease. (The Role of Alveolar and Leukocyte Elastases in the Etiology of Pulmonary Emphysema).

Introduction and Overall Objectives:

Diseases of the lung which cause obstruction to air flow such as pulmonary emphysema, chronic bronchitis and asthma are major causes of morbidity and death in the adult population. At present, we know little of the chemical basis of the pathogenetic processes which produce destruction and alteration of the architecture of the lung. Yet, it is clear that these structural alterations lead to irreversible changes in lung function and severely limit therapeutic effectiveness in these diseases.

Over the past five years, studies from this Laboratory (1-3) and others (4-6) have demonstrated that alteration of lung elastin by elastase leads to marked increases in pulmonary distensibility and in the morphological development of pulmonary parenchymal destruction consistent with pulmonary emphysema. The administration of collagenase produces changes in the tensile strength of lung parenchymal tissue but does not produce pulmonary emphysema (1,4,6). It also is demonstrable that a single exposure of hamster lung in vivo to elastase by the tracheal route leads to subsequent and progressive damage to the alveoli in the following weeks and months leading to the morphological appearance of pulmonary emphysema (7). These observations in experimental preparations have focussed attention on the crucial role of elastin in pulmonary parenchymal tissue to maintain the normal geometric configuration of the alveolus. The structural characteristics of elastin and the relationship of elastin to other connective tissue components such as collagen and glycosaminoglycans which determine the structural role of elastin are beginning to be investigated (8,9). However, such observations indicate the need to understand the chemical and morphological processes by which the initial and the subsequent injury to elastin and the alveolar septal tissue occurs after exposure to elastases.

9. Details of experimental design and procedures (continued)

Leukocytes contain both elastolytic (10) and collagenolytic enzymes (11). The leukocyte elastase is inhibited by alpha$_1$-antitrypsin and Janoff has shown that the inhibitory component for these elastases is missing in the serum of individuals with alpha$_1$-antitrypsin deficiency (12). Similarly, Ohlsson has reported (13) that leukocyte elastase as well as a second neutral leukocyte protease formed alpha$_1$-antitrypsin complexes with characteristic electrophoretic mobility and more recently Ohlsson has demonstrated at least two collagenases and three elastases which have neutral pH optima and are released to a great extent from the leukocytes during phagocytosis of immune complexes or bacteria (14). He has also pointed out that the relative affinity of granulocyte collagenase for alpha$_2$-macroglobulin is about ten times as strong for alpha$_1$-antitrypsin. However, alpha$_1$-antitrypsin is the major serum inhibitor of elastase.

Alveolar macrophage elastase was first described in 1971 by Janoff, Rosenberg and Galdston (15). Human alveolar cells collected by lavage, as well as alveolar macrophages from rabbit lungs, were studied. However, unlike the leukocyte elastase, very little work has been done on the alveolar macrophage elastases. A recent paper by Harris et al. (16) indicates that smokers have a statistically significantly greater concentration of elastase in alveolar macrophages than non-smokers. In contrast, the relative concentrations of leukocyte elastase are the same in both populations. In addition, it has been known for some time (17, 18) that macrophage cell density is increased approximately four times in smokers as compared with non-smokers. The elastase isolated from alveolar macrophages resembles the leukocyte elastase in several respects but may differ in its substrate specificity, i.e., the exact site of cleavage in the elastin molecule as well as its susceptibility to various inhibitors. Although both enzymes are inhibited by alpha$_1$-antitrypsin in the serum, the alveolar macrophage elastase is inhibited to a much lesser extent. Also, a substance present in one molar NaCl extracts of minced human lung tissue inhibits leukocyte but not macrophage elastase (19). Mass, Ikeda, Meranze, Weinbaum and Kimbel (20) have been able to induce lesions resembling human emphysema by intratracheal administration of alveolar macrophages as well as leukocyte proteases in dogs. It is also of interest that significantly higher levels of alpha$_1$-antitrypsin inhibitor have been found in the alveolar macrophages of smokers which suggests that chronically increased amounts could be a protective mechanism against proteolysis (21).

In studies done thus far by the principal investigator and collaborators, the elastase activity of the circulating polymorphonuclear leukocytes has been found to be statistically significantly higher in the population of patients with chronic obstructive lung disease with normal MM alpha$_1$-antitrypsin phenotrype as compared with a population of adult normal subjects (22).

Since the lung normally has a high content of leukocytes which become sequestered in pulmonary capillaries, the alveolar septal area is particularly susceptible to the elastolytic effects of leukocyte lysosomal enzymes. This leukocyte sequestration has been found to be greatly enhanced during endotoxin shock in dogs and has been used as a technique to produce parenchymal destruction from increased elastolytic activity (23, 24). However, the potentially pathogenetic role of the alveolar macrophages which also contain some level of elastolytic activity and which have been shown capable of inducing morphological changes of emphysema in experimental animals resembling the human disease deserves further exploration. These investigations will consider the following:

9. Details of experimental design and procedures (continued)

1. The elastolytic activity of the normal alveolar macrophage in experimental animals and normal man as compared with leukocyte elastolytic activity.

2. The capacity of alveolar as well as leukocyte elastolytic enzymes to increase or decrease in response to specific in vivo and environmental influences.

3. The dynamics of changes in elastolytic activity in both alveolar macrophages and leukocytes with respect to the duration of effects causing increases or decreases in elastase activity in both species of cells.

4. Ultimately, to extend these investigations to include exploration of the inhibitors of both alveolar and leukocyte elastases which are present both in serum and in the alveolus and which may also respond to pathogenetic stimuli.

This study then is designed to explore the role of elastolytic enzymes of the circulating polymorphonuclear leukocyte and the alveolar macrophage in the pathogenesis of obstructive lung disease in man and in experimental animals.

Experimental Plan

The experimental plan involves two major parts: 1) the study of animal alveolar macrophages and circulating polymorphonuclear leukocytes and 2) studies in human subjects.

1. Animal Studies

The objective in this portion of the study is to measure the elastase activity of alveolar macrophages as compared with polymorphonuclear leukocytes in three species of animals, namely, hamsters, rats and dogs. Control measurements will be done in these species in normal animals to allow a series of experimental studies to determine the effect of various interventions on the elastase activity of both alveolar macrophages and polymorphonuclear leukocytes. These interventions are as follows:

A) Tobacco Smoke

The effect of daily exposure to tobacco smoke for a period of 6 to 10 weeks in each species to determine the ability of whole tobacco smoke to bring about an increase in alveolar and/or polymorphonuclear leukocyte lysosomal elastase activity. If alveolar macrophage elastase is increased the study will be continued to determine the rate at which alveolar or polymorphonuclear leukocyte elastase decreases to normal after cessation of exposure to tobacco smoke.

Rats and hamsters (in groups of 10 animals each) will undergo daily exposure to unfiltered cigarette smoke by a smoking machine using a "standard" reference cigarette in amounts of 4 cigarettes per day over a short interval of the day. This exposure has been shown in guinea pigs (25) to be associated with an increase in free lung cells (both macrophages and leukocytes) after 4 weeks. Also, after such exposure, histological effects on the goblet cells and epithelium, as well as increased amounts of mucus, have been described (25).

9. Details of experimental design and procedures (continued)

A) Tobacco Smoke (continued)

These findings in guinea pigs are similar to the response of free lung cells in human smokers as compared with non-smokers (.17, 26).

In later phases of this work, two aspects of smoke exposure in rats will be explored further. 1. The elimination of particulate matter in smoke to determine the effect of the absence of particulates on alveolar macrophage and leukocyte elastase activity and 2. The control of intercurrent pulmonary infection by administration of antibiotics to animals during the period of exposure. In the work of Rylander (25) control of both of these factors resulted in significantly less increase in free lung cells and in the histological evidence of bronchial irritation. It would be of importance to assay concomitant effects of these interventions on alveolar macrophage and leukocyte elastase activity.

B) Proteolytic Enzymes

The effect of an initial proteolytic enzyme injury to the lung on the alveolar and polymorphonuclear leukocyte elastase activity. This portion of the study is being undertaken to examine the mechanism by which a single injury to the lung, in this case proteolytic enzyme injury to the pulmonary parenchyma, results in subsequent and progressive damage to the lung resulting in progression of the pulmonary emphysema in experimental animals after an initial insult. While such a result may occur from mechanical forces, leading to altered connective tissue resistance to tissue stresses, the possibility exists that such initial proteolytic injury may result in subsequent increases in elastase activity of alveolar macrophages or polymorphonuclear leukocytes which then have access to the lung. For these experiments, groups of 20 hamsters, and 10 dogs, will be exposed to papain aerosol intratracheally. Levels of alveolar and polymorphonuclear leukocyte lysosomal elastase activity will be measured in the control state before exposure to the proteolytic enzyme, at 2 days after exposure, and then at weekly intervals over the next 1 month to 6 weeks in each species. If it is determined that elastase activity is increased, measurements will be continued for 2 months at which time the animals will be sacrificed and the lungs examined morphologically and by point counting to quantify the extent of emphysema.

C) Nitrogen dioxide

It has been demonstrated that rats (27) exposed to nitrogen dioxide in the inspired air in amounts of 15 ppm and above for approximately 4 months develop morphological emphysema. The basis of this emphysema is unknown. The potential mechanism for tissue destruction resulting from such exposure is the stimulation of elastase activity in cells which normally carry elastolytic enzymes, such as the alveolar macrophage and circulating polymorphonuclear leukocyte.

Accordingly, rats will be exposed to NO_2 according to the technique of Freeman et al. (28) for a period of approximately 4 months. Alveolar macrophages will be lavaged from these animals at intervals of 3 weeks to determine if there is a significant increase in alveolar macrophage elastase activity as compared with a group of control animals exposed to the same control environment but not exposed to NO_2. Also, circulating polymorphonuclear leukocytes will be harvested from pooled blood samples of 4 rats each and analyzed for

9. Details of experimental design and procedures (continued)

elastase activity. Methods for assay of alveolar macrophages and leukocytes are outlined below. If the elastase activity of one or both of these cell sources is increased, the levels will be measured after cessation of exposure to NO_2 to determine if there is a return to normal.

It is noteworthy that Kleinerman (29,30) has reported the results of exposure of hamsters to NO_2 in concentrations of 45 to 55 ppm for 21 to 23 hours daily for ten weeks. In this species, enlargement of alveolar spaces, epithelial hyperplasia, and abundant inflammatory cells, predominantly neutrophiles and macrophages around respiratory bronchioles and alveolar ducts, were seen but no pulmonary emphysema. It is, therefore, pertinent to compare alveolar macrophage and polymorphonuclear leukocyte elastase activity in the rat and the hamster after similar exposure to NO_2.

As outlined, these experiments are concerned with the response of pulmonary macrophages and leukocytes to inhaled NO_2 with respect to the production of anatomical emphysema. However, the response with respect to cellular elastase activity is of importance whether or not emphysema is produced. The measurement of elastase activity is pertinent to the characterization of the injury produced in the lung by NO_2.

Studies requiring exposure of animals to nitrogen dioxide for prolonged periods to produce emphysema will be carried out in collaboration with Dr. Gustave Freeman of the Stanford Research Institute in Menlo Park, California, to house, expose and monitor the exposure of rats to desired concentrations of nitrogen dioxide as well as ozone and other atmospheric pollutants. Histological characteristics of exposure to nitrogen dioxide, ozone and the combination of the two, as well as the pattern and morphological features of pulmonary emphysema, resulting from exposure to NO_2 or the combination of NO_2 and O_3 have been extensively reported in publications by Dr. Freeman and his co-workers (27,28, 31-34).

Alveolar macrophages and polymorphonuclear leukocytes will be harvested from the experimental animals in Dr. Freeman's laboratory and the lysosomal enzyme preparation carried to the step where it may be held frozen at -20°C for prolonged periods without losing elastase activity. Specimens will be transported by air freight in the frozen state for assay for elastase activity in Dr. Mandl's laboratory.

The precise methods for generating NO_2 of desired concentration, for exposing rats to these concentrations over prolonged periods, have been described previously by Dr. Freeman and co-workers (27,28) and will not be recapitulated here. Dr. Freeman has indicated his willingness to collaborate in these studies and has so written in the attached letter.

As a later phase of this work, it will be possible to examine the effect on alveolar macrophage and polymorphonuclear leukocyte elastase of exposure to ozone in concentrations of from .5 ppm O_3 to .9 ppm O_3 or as a combination of NO_2 and O_3 (.9 ppm O_3 and .9 ppm NO_2) which has been shown to produce emphysema in a shorter interval than with NO_2 alone (29).

These studies of NO_2 and O_3 are of particular significance because NO_2 is produced by tobacco smoke and both are atmospheric pollutants.

9. Details of experimental design and procedures (continued)

Morphological Studies

In each group of experimental animals in this portion of the study, one lung from representative animals will be inflation-fixed in formalin at 25 cm H_2O and sections will be stained and examined for emphysema by microscopic section and the emphysema graded by the point-counting method (35,36).

Parts of the remaining lung will also be prepared for electron microscopy. Alveolar lining cells, including the alveolar macrophages, will be examined by electron microscopy to determine if distinctive morphological alterations occur concomitant with change in alveolar macrophage elastase activity determined chemically. Studies of the ultrastructural characteristics of pulmonary interstitial elastin will also be carried out.

Dr. Joshua Fierer, who will conduct the morphological studies, has had experience over the past three years in the light and electron microscopy of both human and experimental emphysema in dogs and rats with particular interest in the electron microscopic appearance of pulmonary elastin and alveolar cell morphology.

2. Human Studies

In studies done thus far, in this Laboratory, it has been demonstrated that polymorphonuclear leukocytes of patients with obstructive lung disease have a statistically significantly higher activity of polymorphonuclear leukocyte lysosomal elastase than do normal control human subjects (22). However, no studies have been done to investigate the potentially pathogenetic role of alveolar macrophage elastase activity in chronic obstructive lung disease syndromes. Also, it has been demonstrated that there are statistically significant differences in alveolar macrophage esterase activity between normal subjects who are smokers and normal subjects who are non-smokers (16). It, therefore, becomes of some significance in understanding the pathogenetic mechanisms leading to alveolar damage in obstructive lung disease to determine if 1) alveolar macrophages in subjects with obstructive lung disease are significantly higher in elastase activity than normal subjects with an asymptomatic pulmonary status and normal pulmonary function, 2) whether patients with obstructive lung disease syndromes who are smokers and non-smokers have significantly different levels of alveolar macrophage elastase activity and 3) to compare the absolute levels of elastase activity of polymorphonuclear leukocyte lysosomes and of alveolar macrophage lysosomes with respect to elastase activity per cell and per microgram of lysosomal protein.

An added feature of this portion of the study will be an attempt to culture alveolar macrophages harvested from patients in vitro. It has been shown by Bennett (37) and by Soderland and Naum (38,39) that alveolar macrophages lavaged from mouse lung can be maintained in vitro in "standardized conditions" for periods of months. Identification of cells from the lung in culture was done by electron and light microscopy (39). A technique

9. Details of experimental design and procedures (continued)

2. Human Studies (continued)

for separation of alveolar macrophages for cloning has been described and has utilized the special macrophage adhesiveness on plastic surfaces to eliminate other cell types (40,41). The capacity of alveolar macrophages to sustain elastase activity in tissue culture can be determined. Such in vitro preparations of cells will allow the investigation of various factors to ascertain the effect of such agents on the cells directly rather than through the in vivo respiratory system. Such agents are 1) tobacco smoke, 2) O_3 and NO_2, 3) hypoxic gas mixtures and 4) changes in pH of the media from acidosis to alkalosis around the norm of 7.40.

For these studies, subjects with obstructive lung disease syndromes are available in the in-patient and out-patient facilities of the Presbyterian, Harlem and Delafield Hospitals. The technique of pulmonary lavage in patients is outlined below.

All patients with obstructive lung disease who are part of this study will have a complete clinical and physiological evaluation. They will also have phenotyping of serum alpha$_1$-antitrypsin by acid starch gel crossed immunoelectrophoresis. The physiological evaluation will consist of measurements of total lung capacity and its components, arterial blood gas composition, single breath diffusing capacity for carbon monoxide and static recoil pressures of the lung at various lung volumes including total lung capacity. From the clinical appraisal, and these physiological measurements, an attempt will be made to distinguish those patients in whom pulmonary emphysema is a predominant component of their obstructive airway disease in an attempt to define correlations between the occurrence of emphysema and quantitative increase in elastase activity in alveolar macrophages and polymorphonuclear leukocytes.

Alveolar macrophages will be obtained in the course of routine bronchoscopies by fiberoptic bronchoscopy for clinical purposes. To obtain alveolar macrophages from normal human subjects, patients coming to bronchoscopy because of isolated small pulmonary infiltrates on chest x-ray without symptoms of pulmonary dysfunction and with normal pulmonary function by physiological testing will be selected. Such patients are now available in the Pulmonary Disease Section at Presbyterian and Harlem Hospitals. Similarly, patients with obstructive lung disease in various degrees of severity come to bronchoscopy in the course of their disease because of the appearance of new pulmonary densities which require visualization, biopsy or brush biopsy. With the consent of the patient, lavage of aliquots of 50 ml of isotonic saline for a total of 200 to 300 ml of fluid warmed to 37°C, will be used for lavage of alveolar macrophages.

In the technique of pulmonary lavage, described by Finley et al. (42), the Metras balloon-tipped catheter was used to instill and withdraw smaller aliquots of isotonic saline, warmed to 37°C, from certain regions of the lung. In normal subjects, good recoveries of the instilled saline were achieved, whereas in patients with obstructive lung disease, recoveries of fluid were poor because of trapping of fluid in emphysematous areas. The use of the fiber-optic flexible bronchoscope and the introduction of small diameter catheters through the

9. Details of experimental design and procedures (continued)

2. Human Studies (continued)

bronchoscope to much more distal airways than can be achieved by the Metras catheter shoul.d allow recovery of adequate volumes of fluid to promote satisfactory alveolar macrophage yields. If necessary, the patient can change position on the fluoroscopy table to assist drainage of the instilled fluid.

The technique for obtaining and assaying polymorphonuclear leukocyte lysosomal elastase activity is also outlined below. This technique requires approximately 60 ml of venous blood. No difficulty has been experienced in obtaining this volume of blood from patients thus far even for repeated determinations.

The principal investigator at present directs a Program Project from the National Institutes of Health entitled "Chemical Predispositions to Pulmonary Injury." This study includes in large part work on the connective tissue composition of the lung in chronic obstructive lung disease in patients and in experimental emphysema induced in animals by proteolytic enzymes and also in diffuse fibrotic reactions of the lung. These studies are focussed on the elastin, collagen and glycosaminoglycans of the lung in the normal state and in disease and the amino acid composition of these components. The studies outlined in the proposal now being submitted are not being done under the NIH supported work and are not a planned part of that project.

In this Program, so far, tissue culture techniques have been employed for various parts of the study and are carried out by Dr. Mary Parshley of the Department of Pathology here. Cell lines of human and rat lung endothelium and fibroblasts have been established in her laboratory and the techniques now in use would be adapted to growing cell lines of alveolar macrophages.

Techniques for Measurement of Elastase Activity of Alveolar Macrophagesand Polymorphonuclear Leukocytes

The technique for the measurement of polymorphonuclear leukocyte elastase activity has been standardized in this laboratory over the past 2 years. The method involves the drawing of 60 ml of venous blood anticoagulated with citrate and is well tolerated by patients. Blood is mixed with 3% dextran in isotonic NaCl and erythrocytes are allowed to settle. Leukocytes are obtained by centrifugation of the supernates washed in 0.34 M sucrose and a suspension is disrupted mechanically by a vacuum suction through a fine wire screen. The diluted homogenate is then subjected to differential centrifugation and the granular fraction which contains elastolytic activity resuspended in 0.15 M phosphate saline buffer pH 7 and disrupted by freeze-thawing. The elastolytic activity is then measured by an assay developed in our own laboratory using a soluble elastin substrate (43).

9. Details of experimental design and procedures (continued)

Techniques for Measurement of Elastase Activity of Alveolar Macrophagesand Polymorphonuclear Leukocytes (continued)

Several aliquots of 50 ml for a total of 250-300 ml will be used to obtain an adequate number of alveolar macrophages for the measurement of elastolytic activity. In all cases the lavage fluids will be filtered first through gauze, then centrifuged for 30 minutes at 17,000 x g to sediment the macrophage granules. This is a higher speed than that used for leukocyte granule separation and has been recommended by Janoff, Rosenberg and Galdston (15). Total cell counts, differential counts, etc. will have to be completed before this step. All subsequent steps will follow the procedure successfully applied in our laboratory to the isolation of leukocyte elastase. Essentially this involves washing twice with cold 0.34 M sucrose solution, followed by mechanical cell disruption of the sucrose suspension by vacuum suction through a fine wire screen. The granular fraction is then separated by differential centrifugation, first at 900 x g to remove unbroken cells and debris leaving the granules in solution, then at 20,000 x g to sediment the granules from the resuspended supernatant. The precipitate in the form of a small greenish dot contains all the elastolytic activity and is carefully washed and suspended in cold 0.15 M phosphate saline buffer pH 7.0. It is disrupted by 7 cycles of freeze-thawing to liberate the enzyme and centrifuged again at 20,000 x g to remove all debris leaving the lysosomal extract in solution. The material will be stored at this stage at $-70^{\circ}C$ or, if sufficient material is available, subjected to additional purification by affinity chromatography on CNBr activated Sepharose 4B coupled to soluble elastin. Elastase activity will be assayed against solubilized elastin by the method of Keller and Mandl (43) or against the sensitive and specific synthetic substrate succinyl alanine alanine alanine p-nitroanilide introduced by Bieth, Spiess and Wermuth (44). Elastolytic activity is expressed in μ units per μ gram protein determined by the assay of Lowry et al. (45). Since the number of cells being assayed is also known, the results will also be analyzed in terms of elastase activity per unit cell.

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10. Space and facilities available (when elsewhere than item 2 indicates, state location):

Laboratories of Dr. Gustave Freeman
Stanford Research Institute
Menlo Park, California

11. Additional facilities required:

none

12. Biographical sketches of investigator(s) and other professional personnel (append):

see attached

13. Publications: (five most recent and pertinent of investigator(s); append list, and provide reprints if available):

see attached

12. Biographical sketches of investigators :

Gerard M. Turino, M.D., is Professor of Medicine, Columbia University College of Physicians and Surgeons and Director of the Cardiovascular Laboratory of the Presbyterian Hospital. REDACTED graduated from Princeton University (A.B.) in R and from the Columbia University College of Physicians and Surgeons in R Medical Internship and one year of residency in medicine were completed at the Columbia University Division at Bellevue Hospital 1948-1950 and one year of assistant residency in medicine at the Yale-New Haven Community Hospital in 1950-1951. In 1953-1954 he was chief resident in medicine at the Columbia University Division at Bellevue Hospital. From 1951-1953 he was a Captain United States Air Force Medical Corps and served as a professional associate in the Division of Medical Services of the National Research Council, Washington, D. C. He has been on the faculty of Columbia University College of Physicians and Surgeons since 1955. From 1956-1961, he was a senior Fellow of the New York Heart Association and from 1961-1971, a career investigator of the Health Research Council of the City of New York. His research interests during this time have been in cardiorespiratory physiology and diseases of the cardiorespiratory system. 40% of his time is devoted to teaching, clinical work and administration and 60% to research. He is a member of the Editorial Boards of the American Review of Respiratory Disease and the journal, Respiration. He was a member of the Task Force on Obstructive Lung Disease of the National Heart and Lung Institute and Chairman of its subcommittee on "Chemical Structure and Function of the Lung". He is a member of the

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Pertinent Publications:

Turino, G.M., Goldring, R.M. and Heinemann, H.O.: Water Electrolytes and Acid-Base Relationships in Cor Pulmonale. Prog. in Cardiovascular Diseases, 12:167, 1971.

Turino, G.M., Lourenco, R.V. and McCracken, G.H.: Role of Connective Tissue in Large Pulmonary Airways. J. Appl. Physiol., 25:645, 1968.

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Turino, G.M., Rodriguez, J.S., Greenbaum, L.M. and Mandl, I.: Mechanisms of Pulmonary Injury. Am. J. Med. 57:493-505, 1974.

12. Biographical sketches of investigators:

Joshua A. Fierer, M.D., Director of Immunopathology, Assistant Professor of Pathology, Columbia University College of Physicians & Surgeons. REDACTED

REDACTED graduated from Alfred University, Alfred, New York (B.A.) in R and from the State University of New York Downstate Medical Center (M.D.) in R. He was an intern in Surgery in 1963, resident in Surgery in 1964, and Assistant in Surgery in 1965 at the University of Rochester School of Medicine and Strong Memorial Hospital, Rochester, New York. From 1965-1967 he served in the United States Air Force Medical Corps at the School of Aerospace Medicine, Texas, and as Chief of Aeromedical Services, Stewart AFB, New York. He completed a residency in Pathology at Columbia Presbyterian Medical Center, New York, and joined Columbia University College of Physicians and Surgeons as an Instructor in Pathology in 1969. During 1969/70 he was a Guest Investigator in Dr. H. Kunkel's Laboratory at Rockefeller University and since 1970 he has been Assistant Professor of Pathology at Columbia University College of Physicians and Surgeons and Director of Microbiology at The Francis Delafield Hospital Division. In 1972 he received an additional appointment as Assistant Attending Pathologist, Presbyterian Hospital, New York and Director of Immunopathology, Columbia University College of Physicians and Surgeons. He is a member of the

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Pertinent Publications:

Buda, J.A., Suciu-Foca N., Fierer, J.A. and Geering, G.: Induction of Runt Disease in Newborn Rats by Prolonged Antigenic Stimulation with Acellular Tissue Extracts. Transplantation 13:332, 1972.

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12. Biographical sketches of investigators:

Ines Mandl, Ph. D., Associate Professor of Reproductive Biochemistry, R REDACTED received her early education in Austria and at the National University of Ireland, in Cork, Eire, and her M.S. and Ph.D. degrees at the Polytechnic Institute of Brooklyn in R and R respectively. From 1945-1949, she was assistant and coworker of the late Professor Carl Neuberg at New York University and at Interchemical Corporation. Since 1949, she has been at Columbia University College of Physicians and Surgeons, first as chief chemist on an Army sponsored project in the Department of Surgery with Dr. E. L. Howes and Dr. John D. McLennan. From 1956 to 1973 she has been Assistant Professor of Biochemistry assigned first to the Department of Microbiology and, since 1959, to the Department of Obstetrics and Gynecology in charge of the Gynecology Research Laboratories at the Francis Delafield Hospital Division. In 1972 she was awarded a Distinguished Alumnus Award by the Polytechnic Institute of Brooklyn. She is a member of the

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She is also Editor-in-Chief of Connective Tissue Research, An International Journal.

Pertinent Publications:

Manahan, J. and Mandl, I.: Primary Structure of Insoluble Tendon Collagen. I. N- and C- Terminal Positional Analysis of Collagenolytic Breakdown Products of Native Collagen. Arch. Biochem. Biophys. 128:6-18, 1968.

Turino, G.M., Senior, R.M., Garg, B.D., Keller, S., Levi, M.M., Mandl, I.: Serum Elastase Inhibitor Deficiency and Alpha$_1$-Antitrypsin Deficiency in Patients with Obstructive Emphysema, Science 165:709-711, 1969.

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Evans, H.E., Keller, S. and Mandl, I.: Lung Tissue Elastin Composition in Newborn Infants with the Respiratory Distress Syndrome and Other Diseases. J. Clin. Invest. 54:213-217, 1974.

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Parshley, M.S., Sampson, P., Mandl, I. and Turino, G.M.: Production of Acidic Glycosaminoglycans by Normal Rat Lung in Tissue Culture. Abstr. J. Cell Biol. 63:258, 1974.

Mandl, I., Darnule, T., Keller, S. and Fierer, J.A.: Changes With Age in the Microfibrillar Component of Lung Elastin. Abstr. 10th International Congr. Gerontol. 1975.

12. *Biographical sketches of investigators:*

Mary S. Parshley, Ph.D. Research Associate in Pathology and Clinical Fellow in Medicine REDACTED She obtained her A.B. from Smith College in R and M.A. and Ph.D. degrees in anatomy from the University of Pennsylvania in R and R respectively. She was a Finney-Howell Postdoctoral Fellow in Anatomy at the University of Pennsylvania from 1938-1939, following which she joined the Dept. of Pathology of Columbia University College of Physicians and Surgeons as a Research Assistant from 1940-1945, advancing to Research Associate 1945-1949. In 1949 she became a Research Associate in Surgery and in 1962 Asst. Professor of Anatomy. She assumed her current position in 1973. She is a charter member of REDACTED served as an officer of this Association. She is also a member REDACTED REDACTED REDACTED

Pertinent Publications:

Parshley, M.S.: The tissue culture of adult tissues. Chap. 17 in the "Transplantation of Tissues", Vol. II, Lyndon Peer, ed., Williams and Wilkins Co., Baltimore, pp. 593-633, 1959.

Simms, M.S. and Parshley, M.S.: The effect of proteins and amino acids on the growth of adult tissue in vitro. Chap. 7 in "Protein and Amino Acid Nutrition", A. Albanese, ed., Academic Press, N.Y., pp. 143-195, 1959.

Parshley, M.S.: Effect of inhibitors from adult connective tissue on growth of a series of human tumors in vitro. Cancer Res. 25:387-399, 1965.

Parshley, M.S., and Mandl, I.: Inhibition of malignant cells in vitro by a component of normal adult connective tissue. Nature, 203:800, Nov., 1965.

Levi, M.M., Parshley, M.S., and Mandl, I.: Antigenicity of papillary serous cystadenocarcinoma tissue culture cells. Am. J. Obst. and Gyn., 102: 433, 1968.

Martorelli, B., Jr., Parshley, M.S., and Moore, J.G.: Effects of chemotherapeutic agents on two lines of human breast carcinoma cells. Surgery, Gynecology & Obstetrics 127:1006, 1969.

Einbinder, J., Parshley, M.S., Walzer, R. and Sanders, S.L.: Effect of cantharidin on malignant epithelium in tissue culture. J. Invest. Derm. 52:291, 1969.

Parshley, M.S., Sampson, P., Mandl, I. and Turino, G.M.: Production of acidic glycosaminoglycans by normal rat lung in tissue culture. J. Cell Biol. 63:258, 1974.

14. First year budget:

A. Salaries (give names or state "to be recruited") Professional (give % time of investigator(s) even if no salary requested)	% time	Salary	Obligatory Fringe Amount	Total
Gerard M. Turino, M.D.	20			
Joshua A. Fierer, M.D.	20			
Ines Mandl, Ph.D.	10	REDACTED		
Mary Parshley, Ph.D.	10			
Technical				
Ja-Shein Lin, B.S., Sr. Technician	100			
Asha Darnule, M.S., Technician	100	REDACTED		
John Moret, Animal Technician	100			
Mary Lynch, Glassware Washer	33 1/3			
Sub-Total for A		R		
B. Consumable supplies (by major categories)				
Glassware and minor equipment				3,500.
Chemicals				3,500.
Tissue Culture Media and Supplies				2,500.
EM and Photographic Supplies				2,500.
Animals				4,000.
Sub-Total for B		16,000.		
C. Other expenses (itemize)				
Animal Care				2,500
Repair and Maintenance				1,500
Publication Expenses				750
Travel				1,000
Sub-Total for C		5,750.		
Running Total of A + B + C		84,968.		
D. Permanent equipment (itemize)				
Smoking Machine				3,000
Sorvall Centrifuge RC-5 and rotors				4,950
Sub-Total for D		7,950.		
E. Indirect costs (15% of A+B+C)		12,745.		
Total request		105,663.		

15. Estimated future requirements:

	Salaries	Consumable Suppl.	Other Expenses	Permanent Equip.	Indirect Costs	Total
Year 2	R	5,120.	5,130.	- 0 -	13,192.	101,140.
Year 3	R	5,330.	5,540.	- 0 -	14,247.	109,230.

10035460 20

16. Other sources of financial support:
List financial support from all sources, including own institution, for this and related research projects.

CURRENTLY ACTIVE

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates
Chemical Predispositions to Lung Injury.	National Heart and Lung Institute - HL 15832	$1,321,303. (5 years)	May 1, 1973 to April 30, 1978.
Chemical Composition of Elastin, Collagen and Glycosaminoglycans of Lungs in Diffuse Interstitial Fibrosis from Sarcoid and Non-Sarcoid Pulmonary Fibrotic Reactions.	New York Lung Association	$23,000. (1 year)	April 1, 1975 to March 31, 1976.

PENDING OR PLANNED

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates
None			

It is understood that the investigator and institutional officers in applying for a grant have read and accept the Council's "Statement of Policy Containing Conditions and Terms Under Which Project Grants Are Made."

Principal investigator

Typed Name Gerard M. Turino, M.D.

Signature [signature] Date 7/1/75

Telephone REDACTED
Area Code Number Extension

Checks payable to

Trustees of Columbia University

Mailing address for checks

c/o Frederick B. Putney, Ph.D.
630 West 168th Street
New York, New York 10032

Responsible officer of institution

Typed Name Frederick B. Putney, Ph.D.

Title Assistant Vice President for Health Sciences Administration

Signature [signature] Date 9/15/75

Telephone REDACTED
Area Code Number Extension

10035460 21

Addendum 1

Columbia University
College of Physicians and Surgeons
630 WEST 168th STREET
NEW YORK, N.Y. 10032
OFFICE OF THE DEAN

January 25, 1975

Memorandum to the File:

In my absence Ms. Robin George, Director, Grants and Contracts Office of the Health Sciences Faculties of Columbia University is authorized to approve and sign grants and contracts on behalf of Columbia University Health Sciences Faculties.

Frederick B. Putney, Ph.D.
Assistant Vice President for
Health Sciences Administration

10035460 22

#1036 - WILL

1003546023

THE COUNCIL FOR TOBACCO RESEARCH – U.S.A., INC.

June 24, 1975

Grant Application No. 1036

PULMONARY

TO: The committee comprising Drs. Bing, Jacobson and Sommers

Subject: James A. Will, DVM, Ph.D., University of Wisconsin
New application No. 1036
"Morphologic and functional correlations of the APUD cells of the lung"

History:

Was not handled as a case.

Request:

Application No. 1036 requests $24,418 for the first year of a three year project.

Documents Submitted:

1. Application received June 23, 1975 (14 pages including CV^{S} of Drs. Will, Bisgard and Quay).

2. Two abstracts

3. Eleven reprints.

David Stone

DS/lp
Encls.

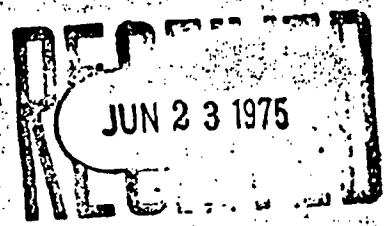
1036

THE COUNCIL FOR TOBACCO RESEARCH–U.S.A., INC.

110 EAST 59TH STREET
NEW YORK, N. Y. 10022
(212) 421-8885

Application for Research Grant
(Use extra pages as needed)

Date:



1. Principal Investigator (give title and degrees):

James A. Will, DVM, Ph.D.
Professor and Chairman

2. Institution & address: Department of Veterinary Science
University of Wisconsin
1655 Linden Drive
Madison, WI 53706

3. Department(s) where research will be done or collaboration provided:

Department of Veterinary Science and Waisman Center

4. Short title of study: Morphologic and functional correlations of the APUD cells of the lung.

5. Proposed starting date: 1 January 1976

6. Estimated time to complete: 3 years

7. Brief description of specific research aims:

a) Define the function of the APUD cells of the lung.

b) Correlate the alterations in function of the lung with morphologic changes in these cells.

8. *Brief statement of working hypothesis:*

Our working hypothesis and supporting evidence are as follows: The Feyrter cells and the NEBs (Neuroepithelial bodies)of the lung have been demonstrated by morphological means to meet the criteria for APUD cells as established by Pearse (1). Current literature supports the facts that these cells are similar to carotid body type I cells, that they are amine containing (2), that the granules within the cells can be depleted by reserpine (3), the fact that they may be modified by acute hypoxia (3,4) and that they have dual innervation and therefore perhaps act as both receptor and effector cells in the airway of the lung (3). By virtue of this evidence the hypothesis is presented that these cells are airway chemoreceptors and perhaps effectors that may influence the regulation of respiration, the control of broncho- and vaso-constriction, and therefore may directly or indirectly play an important role in the development, maintainance, and perhaps pathogenesis of pulmonary hypertension.

9. Details of experimental design and procedures (append extra pages as necessary)

Based on the above hypothesis the following questions can be asked and evidence obtained to support or reject the hypothesis:

1) Does the number and content of these cells remain constant in the change from neonate to the adult?

Moosavi, et al attempted to answer this question in a semi-quantitative way (4). These workers did not do a complete morphometric study. The fact that the number of Feyrter cells decreased per unit of bronchiolar epithelium did not take into consideration the growth of the lung during this 31 day period.

Experiment: We propose to use rabbits rather than rats. Pregnant females will be the adult subjects. Their young will be killed at intervals after birth and complete morphometric studies will be done to quantitate the numbers of these cells per unit of lung volume.

We propose to study the morphology and content of these cells by several techniques: Currently, one graduate student is working in Odense, Denmark with Dr. E. Hage (2) for 8 weeks to perfect techniques in (1) light microscopy and (2) formalin-induced-fluorescence (FIF). Dr. Hage has also agreed to come to this laboratory in September of this year to do a preliminary study with us. Professor Quay, a co-investigator has experience with (3) auto radiography and we plan to use these techniques in his laboratory with labelled amines, polypeptides, their precursors and metabolites. The content of the granules and the cells totally will be assessed by this technique as well as differential fluorescence. Finally, both Professor Quay and Professors Will and Bisgard jointly have postdoctoral fellows working on the (4) electron microscopy of these cells or similar cells. The combination of these four techniques should provide us with the ability to correlate changes in the cells and the granules within the cells with the functional studies we propose.

2) Is the effect of chronic hypoxia similar to that found acutely by Lauweryns, i.e. depletion of granular content consisting principally of serotonin?

Evidence in our own laboratory using methysergide and serotonin in aerosolized form implies that serotonin is not the principal mediator of hypoxic vasoconstriction (5). We used aerosolized compounds as well as those administered IV because of evidence supported by the work of Hauge (6) which makes it likely that the receptors involved in the pulmonary vasoconstrictor response are located closer to the airway than the capillaries. Our work does not exclude the possibility that serotonin may be one of several mediators in the response. Lauweryns supports this by the fact that more than one compound appeared to be present in the cells. We have further demonstrated that animals exposed to chronic hypoxia have an exaggerated response to acute hypoxia (7).

This implies that the mechanism may be different for acute and chronic hypoxia. This is supported by work done by Tucker and Reeves(8). Our studies also show that this change in reactivity is not related entirely to muscular hypertrophy of the pulmonary vasculature, therefore the possibility exists that there is an alteration in lung metabolism initiated or mediated by substances elicited by these cells.

Experiment: A group of pregnant rabbits would be placed in the hypobaric chamber of the Biotron facility here at Madison or taken to our high altitude laboratory at Climax, Colorado (elevation 3400 m., P_{BP}=510 mmHg) and the first experimental protocol would be duplicated.

A further question that would be answered would be: Does exposure to chronic hypoxia cause the number of these cells to increase or have increased activity in the adult as well as the neonatal animal?

3) If these are chemoreceptors do they change in number or content when the carotid bodies and the aortic bodies are denervated?

Work in our laboratory in calves and ponies has demonstrated that animals with the carotid bodies removed and in the case of ponies, the aortic bodies also denervated tend to develop pulmonary hypertension at higher P_{IO_2}s because of the tendency to hypoventilate and develop low alveolar PO_2s (9,10).

Experiment: Although our experience is mainly with the carotid body removal and aortic body denervation in large animals,we propose to use the rabbit for the initial morphologic correlative studies because this species is unique in that it has a separate nerve to the aortic body which can be severed without thoracotomy.

Animals will be prepared and treated in groups similar to experiments 1 and 2.

Once the morphologic studies in experiments 1, 2, and 3 enforce or reject the postulates presented, studies will be extended to larger unanesthetized species like the dog and pony where work will be continuing on ventilatory control. Hopefully dual use can be made of the surgically prepared animals. These animals are much more adaptable to complete functional studies than the rabbit or other laboratory animals, particularly if the studies are performed in the unanesthetized state.

4) If these are chemoreceptors, can they be influenced by the administration of aerosolized compounds which will block, stimulate, deplete, poison, release, or otherwise modify the metabolism of these compounds contained in the granules of these cells?

We have preliminary evidence that low levels of an H_1 blocker that is administered by aerosol modified the vasoconstrictive response to acute hypoxia. This same blocker placed IV required toxic doses that could only be administered to anesthetized animals or in isolated perfused lungs to be effective.

Experiment: Actually this would be a long series of experiments which would hopefully pharmacologically characterize these cells functionally and using the morphological methods developed in earlier experiments to correlate the results. Agents such as α-Methyltyrosine, α and β adrenergic agents, cyanide, doxapram, lidocaine, ethanol, etc., could be used. Complete hemodynamic studies would be done including pressures, cardiac output, oxygen content, heart rate, blood gases, hematocrit, and hemoglobin. Resistances, oxygen transport, work indices, etc. would be calculated. Pulmonary parameters such as ventilation, ventilatory rate,volumes, alveolar gases, compliance can be measured and calculated.

It is obvious that the direction that these studies will go is dependent upon the success and findings of the initial studies.

9. a. BIBLIOGRAPHY

1. Pearse, A.G.E.: The cytochemistry and ultrastructure of polypeptide hormone-producing cells of the APUD series and the embryologic and pathologic implications of the concept. J. Histochem. Cytochem. 17: 303-313, 1969.

2. Hage, Esther: Amine-Handling Properties of APUD-cells in the Bronchial Epithelium of Human Foetuses and in the Epithelium of the Main Bronchi of Human Adults. Acta path. microbiol. scand Section A, Vol. 81: 64-70, 1973.

3. Lauweryns, J. M. and Marnix Cokelaere: Hypoxia-sensitive Neuro-epithelial Bodies; Intrapulmonary Secretory Neuroreceptors, Modulated by the CNS. Z. Zellforsch. 145: 521-540, 1973.

4. Moosavi, H., P. Smith, and D. Heath: The Feyrter Cell in Hypoxia. Thorax, Vol. 28: 729-741, 1973.

5. Ungerer, T., J. A. Orr, G. E. Bisgard and J. A. Will: Hemodynamic Responses in the Pig; The Acute Effects of Serotonin and Hypoxia. Abstract. Presented at 55th Conference of Research Workers in Animal Diseases. Dec. 2-3, 1974. Copy appended to application.

6. Hauge, A.: The pulmonary vasoconstrictor response to acute hypoxia. Prog. Resp. Res. 5: 145-155, 1970.

7. Ruiz, A. V., G. E. Bisgard, I. B. Tyson, R. F. Grover, and J. A. Will: Regional Lung Function in claves during acute and chronic pulmonary hypertension. J. Appl. Physiol., 37: 384-391, 1974.

8. Tucker, A. and J. T. Reeves: Non-sustained pulmonary vasoconstriction during acute hypoxia in anesthetized dogs. Am. J. Physiol. 228: 756-761, 1975.

9. Bisgard, G. E. and Vogel, J. H. K.: Hypoventilation and pulmonary hypertension in calves after carotid body excision. J. Appl. Physiol. 31: 431-438, 1971.

10. Forster, H. V., G. E. Bisgard, B. Rasmussen, D. D. Buss, J. A. Orr, and M. Manohar: The Effect of Peripheral Chemoreceptor Denervation on Ventilatory Acclimatization to Hypoxia in Ponies. Abstract. To be presented to APS Fall Meeting, Oct. 5-11, 1975. Copy appended to application.

10. Space and facilities available (when elsewhere than item 2 indicates, state location):

A complete cardiopulmonary laboratory is available under the direction of Professors Will and Bisgard. A departmental laboratory operates to provide histological tissue preparations. A tissue freeze drier was obtained last year. Electron microscopes are available in the Department of Veterinary Science, the Muscle Biology Institute and the Waisman Center.

Professors Bisgard and Will in cooperation with Dr. R. F. Grover, University of Colorado Medical Center have a research laboratory at Climax, Colorado. There are living quarters, a corral for large animals and a laboratory building. This facility provides an ideal situation for large animal studies that can't be accommodated in a hypobaric chamber or for small animal experiments that have too many animals to fit into the chamber or are of such a long term that it is too costly to do in the chamber.

Professor Quay has a laboratory equipped to do autoradiography.

Animal rooms are available for large and small animals.

11. Additional facilities required:

Funds were asked for animal cages. While rooms and animal care is available, there is a shortage of cage facilities for rabbits.

12. Biographical sketches of investigator(s) and other professional personnel (append):

13. Publications: (five most recent and pertinent of investigator(s); append list, and provide reprints if available).

12. a.

BIOGRAPHICAL SKETCH

Name: James Arthur Will, D.V.M. Address: REDACTED
REDACTED
REDACTED

Born: REDACTED Birthplace: REDACTED

Citizenship: REDACTED Marital Status: REDACTED

EDUCATION:

High school graduated from: Wauwatosa High School Year: R

University Attended	Dates Attended	Degree	Date	Major and Minor
University of Wisconsin	REDACTED	B.S.	R	Agriculture Animal Husbandry
University of Wisconsin	REDACTED	M.S.	R	Animal Science Genetics
Kansas State University	REDACTED	D.V.M.	R	Veterinary Medicine Pathology
University of Wisconsin	REDACTED	Ph.D.	R	Veterinary Science Comp. Cardiology

MILITARY SERVICE:

Branch: Army of the United States From: Feb. 1954 to Feb. 1956
QMC, 1st Lt.

POSITIONS:

1952-53 Research Assistant, Department of Meat and Animal Science, University of Wisconsin, Madison, Wisconsin.

1956-60 Research Assistant, Kansas State University, Manhattan, Kansas.

1960-61 Practicing Veterinarian, Columbus Veterinary Hospital, Columbus, Wisconsin.

1961-64 Practicing Veterinarian, Self-employed, Columbus, Wisconsin.

1964-67 Postdoctoral Research Fellowship, University of Wisconsin, Department of Medicine, Cardiovascular Research Laboratory and Department of Veterinary Science - NIH (Candidate for Ph.D.) (3 years) December, 1967.

1966 Consulting veterinarian, Avian Pathology.

1003546030

1967 Assistant Professor of Veterinary Science and Assistant Scientist, Cardiovascular Research Laboratory, Department of Medicine, Medical School.

1971-74 Associate Professor of Veterinary Science, College of Agricultural and Life Sciences and Department of Medicine, Medical School.

1974 to present Professor and Chairman of Veterinary Science, College of Agricultural and Life Sciences and Staff member, Cardiovascular Research Laboratory.

12. a. (continued)

BIOGRAPHICAL SKETCH (continued)

1972-73 Special Fellow, NHLI, Department of Pathology, New Medical School, University of Liverpool, Liverpool, England.

PROFESSIONAL SOCIETIES:

REDACTED

REDACTED

REDACTED

REDACTED

HONORS RECEIVED:

NIH Postdoctoral Fellow. 1964-1967.

Burr Beach Award. 1967.

NIH Special Postdoctoral Fellow. 1972-1973.

SPECIAL APPOINTMENTS:

Member, World Health Organization Committee for meeting on "Primary Pulmonary Hypertension in Man." October 15-17, 1972, Geneva, Switzerland.

12. b.

BIOGRAPHICAL SKETCH

Name: Gerald Edwin Bisgard Birth Date: REDACTED

Present Home Address: REDACTED Place of Birth: REDACTED

Education:

Denver Public Schools, Graduated South High School - REDACTED

REDACTED

College Education:

B.S.	Biological Science, Colorado State University -	
D.V.M.	Veterinary Medicine, Colorado State University -	REDACTED
M.S.	Clinical Medicine, Purdue University -	R
Ph.D.	Veterinary Science (Physiology), University of Wisconsin	REDACTED

College Honors:

Sigma Xi
Beta Beta Beta
Omicron Delta Kappa
Phi Zeta
Graduation "With Distinction" (D.V.M.)

Professional Societies:

REDACTED

REDACTED

REDACTED

Positions Held:

1962-1967	Instructor in Veterinary Clinics, Purdue University.
1967-1969	Assistant Professor in Veterinary Clinics, Purdue University.
1967-1968	On Leave of Absence from Purdue University for Special Fellowship in Research at Cardiovascular Pulmonary Laboratory, University of Colorado Medical Center.
1969-1971	Special Fellowship in Research and Ph.D. Program, University of Wisconsin, Cardiovascular Laboratory, Department of Medicine and Department of Veterinary Science.
1971-1974	Assistant Professor of Veterinary Science, University of Wisconsin
1974-	Presently Associate Professor of Veterinary Science, University of Wisconsin

1003546032

12. c

BIOGRAPHICAL SKETCH

Name: Wilbur Brooks Quay REDACTED

Title: Professor in Waisman Center and Department of Zoology

Role in proposed project: Co-investigator

Birthdate: REDACTED

Nationality: REDACTED

Education:

Institution	Degree	Year	Field
Harvard College	A.B. (magna)	R	Biology
University of Michigan	M.S., Ph.D.	R	Zoology
Netherlands Central Institute for Brain Research	Res. Fellow	R	Brain and Neuroendo. Res.

Honors:

Phi Beta Kappa - Harvard, 1950; Sigma Xi - University of Michigan, 1952; Horace H. Rackham Predoctoral Fellow - University of Michigan, 1951-52; Professorships in Miller Institute for Basic Research in Science - University of California, Berkeley, 1964-65, 1971-72; various lectureships.

Major Research Interest: Central nervous system and neuroendocrinology; regulatory mechanisms and chronobiology.

Research and Professional Experience:

Professor, Department of Zoology, and Waisman Center, University of Wisconsin, Madison, Wisconsin. June, 1973 - present; research and teaching (undergrad. and grad. courses, train predoctoral and post-doctoral students).

Professor, Department of Zo-logy, University of California, Berkeley, 1967-1973.

Fellow, Netherlands Organization for the Advancement of Pure Research, at Netherlands Central Institute for Brain Research, Amsterdam.

Associate Professor, Department of Zoology, University of California, Berkeley, 1961-1967.

Assistant Professor, Department of Zoology, University of California, Berkeley, 1956-1961.

Instructor, Department of Anatomy, University of Michigan Medical School, 1952-1956.

Teaching Fellow, University of Michigan, Ann Arbor, spring 1950-51.

Teaching Assistant, University of Michigan, Ann Arbor, fall 1950-51.

Research Assistant, University of Michigan, Ann Arbor, 1949-1950.

10035460 33

13. a.

1. B. E. McKenzie, B. C. Easterday and J. A. Will. Light and Electron Microscopic Changes in the Myocardium of Influenza Infected Turkeys. Amer. J. Path., Vol. 69, No. 2, Nov. 1972, pp. 239-254.

2. A. V. Ruiz, G. E. Bisgard, I. B. Tyson, R. F. Grover, and J. A. Will: Regional Lung Function in Calves During Acute and Chronic Pulmonary Hypertension. J. Appl. Physiol., 37(1974): 384-391.

3. J. A. Will and J. M. Kay. Hypertensive Pulmonary Vascular Disease Associated with Papain Emphysema in Rats. Respiration 31 (1974): 208-220.

4. Allin, E. F., Miller, J. M., Rowe, G. G. and J. A. Will: Effects of Intraperitoneal Administration of Proprandol on the Mouse Heart: Histochemical and Electron Microscopic Observations. Am. J. Card. 33 (May 6, 1974): 639-642.

5. Bisgard, G. E., J. A. Will, I. B. Tyson, L. M. Dayton, R. R. Henderson and R. F. Grover: Distribution of regional lung function during mild exercise in residents of 3100 m. Res. Physiol. 22(1974): 369-379.

13. b.

1. Bisgard, G. E., A. V. Ruiz, R. F. Grover and J. A. Will. Ventilatory control in the Hereford calf. J. Appl. Physiol. 35:220-226, 1973.

2. Ruiz, A. V., G. E. Bisgard, and J. A. Will. Hemodynamic responses to hypoxia and hyperoxia in calves at sea level and altitude. Pflugers Arch (European J. Physiol), 344:275-286, 1973.

3. Bisgard, G. E., A. V. Ruiz, R. F. Grover and J. A. Will. Ventilatory acclimatization to 3400 meters altitude in the Hereford calf. Respiration Physiol. 21:271-296, 1974.

4. Bisgard, G. E., J. A. Orr and J. A. Will. Hypoxic pulmonary hypertension in the pony. Am. J. Vet. Res. 36, No. 1 (Jan., 1975): 49-52.

5. Rawlings, C. A., M. L. Birnbaum, and G. E. Bisgard. Static pulmonary compliance in ponies. J. Appl. Physiol., 38(4):657-660, 1975.

13. c.

1. 1974. (Quay, W. B.) Pineal canaliculi: demonstration, twenty-four-hour rhythmicity and experimental modification. American Journal of Anatomy. 139(1): 81-93.

2. 1974. (Quay, W. B.) Pineal Chemistry: In Cellular and Physiological Mechanisms. Charles C. Thomas Publisher, Springfield, Illinois, pp. i-xv, 1-430, 68 Figures, 91 Tables.

3. 1973. (Lew, Gloria M., and W. B. Quay) Circadian rhythms in catecholamines in organs of the golden hamster. American Journal of Physiology. 224: 503-508.

4. 1973. (Lew, Gloria, and W. B. Quay) The mechanism of circadian rhythms in brain and organ contents of norepinephrine: Circadian changes in the effects of methyltyrosine and 6-hydroxydopamine. Comparative and General Pharmacology. 4(16): 375-382.

5. 1972. (Quay, W. B.) Pineal vasoconstriction at daily onset of light: its physiological correlates and control. The Physiologist. 15(3): 241.

14. First year budget:

A. Salaries (give names or state "to be recruited")	% time	Amount
Professional (give % time of investigator(s) even if no salary requested)		
James A. Will, Principal Investigator	30	
Gerald E. Bisgard, Co-investigator	10	
Wilbur B. Quay, Co-investigator	5	
Aureliano Hernandez, Research Assistant	50	
Barbara A. Meyers, Research Assistant	50	
Technical		
Lab Tech II - To be recruited ($8,124 plus fringe benefits $2,063)	100	$10,187
Sub-Total for A		$10,187
B. Consumable supplies (by major categories)		
Radioisotopes		1,500
Animals and animal care		1,500
Functional study supplies: Cardiogreen, recording paper, surgical supplies, etc.		1,500
Morphology study supplies: EM, auto radiography, FIF		1,500
Sub-Total for B		6,000
C. Other expenses (itemize)		
Biotron rental of hypobaric chamber, 90 days @ $15.30/day		1,377
Meeting travel		1,000
Travel to high altitude laboratory and living expenses		1,000
Equipment repair		500
Publishing costs cnt		300
Sub-Total for C		4,177
Running Total of A + B + C		20,364
D. Permanent equipment (itemize)		
Animal cages		1,000
Sub-Total for D		1,000
E. Indirect costs (15% of A+B+C)		3,054
Total request		24,418

15. Estimated future requirements:

	Salaries	Consumable Suppl.	Other Expenses	Permanent Equip.	Indirect Costs	Total
Year 2	10,798	7,500	5,500	1,000	3,570	28,368
Year 3	11,445	8,500	6,000	1,000	3,892	30,837

10035460037

16. Other sources of financial support:

List financial support from all sources, including own institution, for this and related research projects.

CURRENTLY ACTIVE

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates
1. Chemoreceptors and Ventilatory Control (Bisgard and Will)	NIH, HL 15473	$69,000	9/1/73 to 8/31/76
2. Mechanisms of Pulmonary Hypertension (Bisgard and Will)	UW # 133-A280 Wis. Heart Ass.	7,500	7/1/75 to 6/30/76
3. Morphologic and Functional Correlations of the APUD Cells of the Lung (Will and Bisgard)	UW Grad. School # 160243	7,344	7/1/75 to 6/30/76
4. Circadian Phase Shifts and Mental Health (Quay)	NIH, MH 22305-01	30,000/yr	6/1/73 to 5/31/77

PENDING OR PLANNED

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates

It is understood that the investigator and institutional officers in applying for a grant have read and accept the Council's "Statement of Policy Containing Conditions and Terms Under Which Project Grants Are Made."

Checks payable to
Board of Regents of the University of Wisconsin System

Mailing address for checks
750 University Avenue
Madison, WI 53706

Principal investigator
Typed Name James A. Will
Signature James A. Will Date 11 June 75
Telephone (608) 262-3177 212
Area Code Number Extension

Responsible officer of institution
Typed Name Robert W. Erickson
Title Director, Research Administration-Financial
Signature Robert W. Erickson Date 6/19/75
Telephone REDACTED
Area Code Number Extension

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MISCELLANEOUS

1003546039

#1039-KULLANDER

[redacted]

1003546040

THE COUNCIL FOR TOBACCO RESEARCH–U.S.A., INC.

June 27, 1975

Grant application No. 1039

MISCELLANEOUS

To: The committee comprising Drs. Gardner, Jacobson and Sommers

Subject: Stig Kullander, M.D., University of Lund, Sweden
New application No. 1039
"Influence of smoking on human foetal growth and post-natal development and on fibrinolysin in the blood of pregnant women. Accumulation and/or damage to human placental and foetal lung tissues of nicotine".

History

Application after personal contact with Dr. Gardner.

Request

Application No. 1039 requests 141.455 Swedish Kr. (approx. $26,040) for the first year of a three year project.

Documents submitted (attached)

1. Application dated June 23, 1975 (6 pages).
2. One reprint.
3. One booklet (departmental publication).

D.S.

DS:wg

#1039

THE COUNCIL FOR TOBACCO RESEARCH–U.S.A., INC.
110 EAST 59TH STREET
NEW YORK, N. Y. 10022
(212) 421-8885

Application for Research Grant
(Use extra pages as needed)

Date: 1975 06 23

1. Principal Investigator (give title and degrees): Stig Kullander, M.D. Professor and Chairman of Obst. and Gyn., University of Lund, Lund, Sweden

Collaborators:
Bengt Källén, M.D. Professor of Embryology
Birger Åstedt, M.D. Assoc. Prof. Obst. Gyn.
Gerhard Genser, M.D. Assoc. Prof. Obst. Gyn.

2. Institution & address:

University of Lund, Lund, Sweden

3. Department(s) where research will be done or collaboration provided:

Dpt. of Obst. Gyn, Malmö General Hospital, Malmö, Sweden
Dpt. of Embryology, University of Lund, Lund, Sweden

4. Short title of study: Influence of smoking on human foetal growth and post-natal development and on fibrinolysin in the blood of pregnant women. Accumulation and/or damage to human placental and foetal lung tissues of nicotine.

5. Proposed starting date: Jan 1. 1976.

6. Estimated time to complete: 3 years.

7. Brief description of specific research aims:
In an earlier prospective epidemiological investigation in Malmö a negative influence of smoking was shown on the weight of the children and placentae and the head circumference of the children. An increased perinatal mortality was found, due to premature placental separation with general fibrinolysin and done to increased occurrence of respiratory diseases of the new-borns. The aims of our new project are to study those findings more in detail.

8. Brief statement of working hypothesis:

Nicotine may accumulate and/or damage enzyme systems in the placental tissue responsible for production of placental fibrinolytic inhibitors and pass through the placenta to damage the production of surfactant principles in the foetal lung tissues.

A more detailed prospective study on smoking pregnant women (Increase of biparietal head diameter measured with ultrasonic during different gestational months. Correlation to placental hormone production - HPL) and follow-up of the children, also using the mothers who stop or take-up smoking as controls during their next pregnancies would allow more firm conclusions regarding the influence of smoking as such.

9. Details of experimental design and procedures (append extra pages as necessary)

A pregnant woman is protected from bleedings by characteristic changes in the coagulation mechanism and in the fibrinolytic system. Some of the coagulation factors, protrombin, fibrinogen and factor VIII, increase during pregnancy. On the other hand there is a decrease in the fibrino-lytic activity. It is well known that the fibrinolytic activity of the blood decreases and that this activity is hardly measurable at term. Also the content of fibrinolytic activators in the vessel walls have been found to be low. This decrease of the fibrinolytic activity returns to normal levels after delivery of the placenta.

Interestingly also high amounts of the fibrinolytic inhibitors have been found to be contained in the placenta. Physiological significance of these inhibitors are presumably to prevent fatal bleedings from the placenta during pregnancy.

Smoking has been found to some extent increase fibrinolytic activity of the blood but only non-pregnant subjects have been studied so far). Hypothetically, this would increase the risk of bleedings from the placenta during pregnancy. It would therefore be of interest to investigate the influence of nicotine on the inhibitors contained in the placenta and the influence of smoking on the fibrinolytic activity of the blood during different stages of pregnancy and in the puerperium (in the same woman). Inhibitors of the placenta were first described by Kawano et al. (Nature 217, 253, 1968) and Abildgaard and Uszinsky (Thrombosis, Diathesis Haemorrhagica 25, 580, 1971). These latter authors separated two fibrinolytic inhibitors from the human placenta. The inhibitory effect of placenta on activators released from the vessel walls has been studied in tissue culture by Åstedt et al. (Proc. Soc. Exp. Biol. Med. 139, 1421, 1972).

Our aim is to: 1) Further separate and characterize the placenta inhibitors produced in tissue culture of the placenta, further to study in tissue culture, the influence of nicotine on the production and release of the inhibitors from the placental tissue. 2) To undertake a detailed clinical study of the fibrinolytic and the inhibitory capacity of the blood in pregnant smokers compared to non-smokers.

Malmö is an ideal town for epidemiological prospective studies. 1/4 mill. of inhabitants. All deliveries are in one Dpt. of Obst. and all pregnant women are coming for prenatal routine ultrasonic screening. The population is stable and all children are followed and studied at 1 and 5 years of age in one Childrens Health Clinics.

cont. nr. 9

The detailed prospective study intended could easily be included in the routine clinical work and only some extra staffpersons must be recruited for secretarial statistical and data processing help.

We have found in Malmö in preliminary studies that nude hairless mice (without thymus) accept human foetal lung tissue. It will grow subcutaneously and its alveoli will be expanded by secretion. Injecting C_{14} nicotin to the mice - also carrying at other subcutaneous places control tissues (placenta, foetal liver and ovary) - would allow a study (liquid scintillation, EM combined with autoradiography) of accumulation and/or damage of nicotine to the human foetal lung and its production of surfactant lamellar bodies.

10. Space and facilities available (when elsewhere than item 2 indicates, state location):

Space and facilites are available for establishing and maintaining a colony of nude mice. A tissue culture laboratory belongs to our Clinic. We have close cooperation with the Blood Coagulation Laboratory of the Hospital and an EM division at the Zoo-physiological Dpt.

11. Additional facilities required:

None.

12. Biographical sketches of investigator(s) and other professional personnel (append):

13. Publications: (five most recent and pertinent of investigator(s); append list, and provide reprints if available).

Included is a recent report from our Dpt. with publication lists.

6/26/75
5.43 Swedish Kr = $1.00

14. First year budget:

A. Salaries (give names or state "to be recruited") Professional (give % time of investigator(s) even if no salary requested)	% time	Amount
Technical		Swedish Crowns
Secretary, to be recruited	100	50.000:-
Laboratory assistant, to be recruited (tissue culture)	100	48.000:-
Scientific assistant, to be recruited (nude mice breeding and operation)	25	13.700:-
Sub-Total for A		111.700:-
B. Consumable supplies (by major categories)		
Mice food		5.000:-
Sub-Total for B		5.000:-
C. Other expenses (itemize)		
Carrel flashs 200		3.000:-
Sephadex G 100 and columns (SR 25/100)		1.000:-
Sub-Total for C		4.000:-
Running Total of A + B + C		120.700:-
D. Permanent equipment (itemize)		
Gel-el-fores-apparatus		2.650:-
Sub-Total for D		2.650:-
E. Indirect costs (15% of A+B+C)		18.105:-
Total request		141.455:-

15. Estimated future requirements:

	Salaries	Consumable Suppl.	Other Expenses	Permanent Equip.	Indirect Costs	Total
Year 2	117.700	5.000	4.000	0	18.105	138.805
Year 3	117.700	5.000	4.000	0	18.105	138.805

16. Other sources of financial support: None.

List financial support from all sources, including own institution, for this and related research projects.

CURRENTLY ACTIVE

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates

PENDING OR PLANNED

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates

It is understood that the investigator and institutional officers in applying for a grant have read and accept the Council's "Statement of Policy Containing Conditions and Terms Under Which Project Grants Are Made."

Principal investigator

Typed Name Stig Kullander

Signature Stig Kullander Date 19750623

Telephone 040/990000
Area Code Number Extension

Checks payable to

Mailing address for checks

Responsible officer of institution

Typed Name

Title

Signature Date

Telephone
Area Code Number Extension

10035460472

#1040-PIERCE

1003546048

THE COUNCIL FOR TOBACCO RESEARCH–U.S.A., INC.

July 2, 1975

Grant Application No. 1040

MISCELLANEOUS

TO: The committee comprising Drs. Feldman, Gardner, Huebner and Wyatt

SUBJECT: Carl W. Pierce, M.D., Ph.D., Harvard Medical School, Boston
New application No. 1040
"Biology of Suppressor T Cells"

History

An informal inquiry was handled as case No. 323 and encouraged.

Request

Application No. 1040 requests $45,339 for the first year of a three year project. Estimates for the second and third years are $58,078 and $62,435, respectively.

Documents Submitted (attached)

1. Application dated June 23, 1975 (26 pages, including CV^{s} of Drs. Pierce, Tadakuma and Peavy).

2. Four publications.

3. Two manuscripts in press.

4. Two manuscrips submitted to press.

David Stone

DS/lp
Encls.

1003549049

1040

THE COUNCIL FOR TOBACCO RESEARCH–U.S.A. INC.

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Application for Research Grant
(Use extra pages as needed)

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3. Department(s) where research will be done or collaboration provided:

Department of Pathology

4. Short title of study:

Biology of Suppressor T Cells

5. Proposed starting date: January 1, 1976

6. Estimated time to complete: 3 years

7. Brief description of specific research aims:

The understanding of physiologic mechanisms which regulate immune responses is a prerequisite for understanding the pathogenesis of disease processes with immunologic components and the effects of environmental factors, such as cigarette smoking, on the immune mechanism. We propose to investigate the mechanisms by which suppressor T cells and their products regulate the development and expression of humoral and cell-mediated immune responses in tissue culture systems. Initially, concanavalin A-activated suppressor T cells and the biologically active mediators they secrete will be used to probe non-antigen-specific regulatory mechanisms. Experimental systems for activation of antigen-specific suppressor T cells and secretion of antigen-specific factors will be developed. These cells and their products will be used in various experimental situations to define the critical parameters which permit selective suppression of a) humoral immune responses without compromising cell-mediated immune responses to the same antigenic determinants, and b) the reverse situation, suppression of cell-mediated immune responses without affecting antibody responses. After these parameters have been defined in tissue culture systems, this information will be applied to relevant animal models.

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8. Brief statement of working hypothesis:

Activation of the complex, multicellular immune mechanism of mammals by foreign antigen results in the development of humoral and cell-mediated immune responses which may either protect the host from invasion by infectious organisms and neoplastic cells or be responsible for rejection of potentially beneficial tissue transplants and the pathogenesis of several disease processes (1). Cigarette smoking has been implicated in the etiology and pathogenesis of a number of respiratory diseases in man (2). Further, cigarette smoking in man and chronic exposure of laboratory animals to cigarette smoke has been associated with impaired humoral (3-9) and cell-mediated (10,11) immune responses and decreased phagocytic and bacteriocidal activity of alveolar macrophages (12-16). The cells of the immune system survive poorly after acute exposure to cigarette smoke *in vitro* (17), but chronic exposure *in vivo* leads to an accumulation of alveolar macrophages resistant to the toxic effects of tobacco smoke but with undetermined functional capacity (8,16,18). However, these effects of cigarette smoke on the cells and responses of the immune system cannot be adequately evaluated until the immune mechanism itself is better understood. This proposal is directed to gaining a better understanding of one aspect of the immune system: the physiologic mechanisms which regulate development and expression of immune responses.

9. Details of experimental design and procedures (append extra pages as necessary)

RESEARCH PROPOSAL

I. Background Information

A. Studies of Other Investigators. Two distinct types of antigen-specific lymphocytes are the precursors of the immune effector cells. In humoral immune responses, the precursors of antibody-producing cells, B cells, respond to antigenic stimulation by differentiating into plasma cells which secrete antibody molecules specific for the stimulating antigen (1, 19, 20). In cell-mediated immune responses, thymus-derived lymphocytes, T cells, after stimulation by cell membrane antigens, may develop into cytotoxic lymphocytes which mediate the various rejection phenomena (allograft and tumor rejection, and graft-versus-host responses) (1, 21-23). Antigen or mitogen activated T cells may also secrete a variety of biologically active mediators (including macrophage migration inhibitory factor (MIF), chemotactic factors, lymphotoxins, skin reactive factors, and interferon) which play a part in the inflammatory processes of delayed hypersensitivity reactions, cytotoxicity to tumor cells, resistance to certain infectious organisms, and activation of macrophages (1, 24-26). In addition, both T and B cells may be rendered tolerant or unresponsive after interaction with antigen under appropriate circumstances (27).

A third, non-specific accessory cell, the macrophage, has important functions in uptake, catabolism and presentation of antigen to T and B cells so crucial in the initiation of immune responses and avoidance of tolerance induction (28). These cells may also act as non-specific effector cells in the expression of both humoral and cell-mediated immune responses (24-26, 28).

T cells are also the critical regulators of the development and expression of both cell-mediated and humoral immune responses (1, 20, 23, 29-31). In a positive regulatory capacity, T cells may function as "helper cells" for development of antibody responses by B cells to complex, multideterminant antigens (T cell-dependent antigens) (1, 20, 29-31), or as "amplifier cells" for development of cell-mediated immune responses, such as graft-versus-host and cytotoxic lymphocyte responses, by other T cells (22, 23, 30, 31). In a negative regulatory capacity, suppressor T cells have been implicated in the regulation of development and expression of most T cell and B cell responses to antigen (23, 27, 29-31). The ubiquitous nature of suppressor T cells is illustrated

by their involvement in: a) many of the phenomena of immunologic tolerance (27); b) antigenic competition (32); c) regulation of IgE antibody responses (33); d) regulation of antibody responses to "T cell-independent antigens" (34); e) chronic allotype suppression (35); f) regulation of antibody responses controlled by histocompatibility-linked immune response genes (36); g) regulation of mixed lymphocyte, cytotoxic lymphocyte, and graft-versus-host responses (23); h) regulation of development and expression of delayed hypersensitivity responses (27, 29-31, 37-39); and i) regulation of development of autoimmune diseases (40, 41).

Suppressor and helper T cells appear to be functionally distinct subsets of T cells rather than a single population of cells whose regulatory function reflects a critical stage of activation (23, 27, 29-31, 42). Suppressor T cells appear to be relatively immature cells which have abundant θ antigen and are functionally short-lived, spleen-seeking, and, in many instances, radiosensitive. In contrast, helper T cells appear to be more mature cells which have less θ antigen and are functionally long-lived, lymph node-seeking, and radioresistant, at least after priming with antigen.

The regulatory activities of T cells may be antigen-specific, e.g., carrier-specific helper or suppressor functions (20, 27, 29-31, 33, 43), or, although T cells are activated by specific antigen, their effects on immune responses may be non-specific, e.g., antigenic competition and the "allogeneic effect" (27, 29-32, 44-46). Lastly, phytomitogens non-specifically activate T cells whose enhancing or suppressing effects on immune responses are also non-specific (42, 47-50). Soluble products released by activated T cells mediate the regulatory functions in some immune responses (20, 27, 29-33, 43-46, 50-52), whereas regulation of other responses may require direct cell-to-cell contact (20, 23, 27, 29-31, 49). Some of the soluble products of activated T cells have been partially characterized. In one system, the mediator of both helper and suppressor functions appears to be a monomeric IgM-like molecule, IgT, produced by T cells (52); a similar molecule appears to mediate only carrier-specific helper functions in another system (33). Further, other investigators have found that T cell factors with different physicochemical properties are involved in the non-specific enhancement of IgG and IgE antibody responses (46). Another class of mediators appears to be a product(s) of the K and/or I regions of the major histocompatibility complex (33, 43, 45, 53). These mediators may lack antigen specificity (45, 53), or have specificity for the carrier moiety of the immunogen (33, 43) and mediate both helper (43, 45, 53) and suppressor functions (33) in different experimental systems. T cells or their products may act directly on potentially responsive T or B cells, or indirectly on macrophages by mechanisms which are still altogether unclear (23, 27, 29-31, 33, 46, 52).

Although much of the phenomenology of helper and suppressor T cells has been recognized and described, our understanding of the precise mechanisms by which these cells and their products operate is still quite obscure.

B. Studies from our Laboratory. Several years ago, experiments were initiated to investigate the immunological consequences of T lymphocyte activation. Since antigen activates only those relatively few T cells having membrane receptors specific for that antigen, the plant lectin and mitogen concanavalin A (Con A) was used to non-specifically activate larger numbers of T cells. Primary IgM and IgG plaque-forming (PFC) responses to sheep erythrocytes (SRBC) by mouse spleen

cells *in vitro* were profoundly suppressed by mitogenic concentrations (1 µg/ml) of Con A added at culture initiation (54). In addition, mitogenic concentrations of Con A suppressed the generation of cytotoxic lymphocytes (CL) in mixed lymphocyte cultures (MLC) of mouse spleen cells (49). The precise mechanisms by which Con A affects immune responses remain to be determined, but multiple mechanisms appear to be involved (55). However, the observation that spleen cells from mice injected with Con A suppressed PFC repsonses by normal spleen cells *in vitro* (54) prompted us to investigate whether one mechanism of Con A-mediated suppression was the activation of a population of suppressor T cells.

Small numbers of mouse spleen or lymph node cells, but not thymus cells, after incubation with 1 µg of Con A/ml for 48 hrs, profoundly suppressed PFC responses to SRBC(48), GAT(synthetic random terpolymer of L-glutamic acid^{60}-L-alanine^{30}-L-tyrosine^{10}), and the T cell-independent antigens, DNP-Ficoll and TNP-lipopolysaccharide (56), and CL responses to alloantigens generated in MLC (49) by normal spleen cells. Other investigators have shown that Con A-activated spleen cells suppress DNA synthetic responses in MLC (57) and, depending on the experimental circumstances suppress or enhance PFC responses *in vitro* (42, 47, 50, 58, 59). The suppression of these immune responses was not antigen-specific and was not mediated by transferred Con A or non-specific cytotoxicity on responding spleen cells as determined by viable cell recovery. The suppression by Con A-activated spleen cells was mediated by T cells, since treatment of spleen cells with anti-θ serum and complement, either before or after activation with Con A, eliminated suppressor cell activity (48, 49). Further, X-irradiation (2000 R) of spleen cells before activation with Con A abrogated generation of suppressor T cells, whereas after activation, the function of suppressor T cells was radioresistant. Suppression of PFC and CL responses was critically dependent on both the numbers of Con A-activated cells added and the time of addition to the responding cultures. Decreasing the number of Con A-activated cells below 10% of the number of responding spleen cells resulted in progressively less suppression, whereas increasing the number over 20% often resulted in non-specific cytotoxicity. Suppression of PFC responses was observed when suppressor T cells were added during the first 48 hrs of culture, whereas CL responses were most efficiently suppressed when Con A-activated cells were added during the first 24 hrs of culture (48, 49).

The most interesting aspect of the suppression of PFC and CL responses by Con A-activated suppressor T cells, and one which may provide a clue to their mechanism(s) of action, is their effect on the kinetics of development of these responses. Kinetic analysis of PFC responses to SRBC revealed that the 90% or greater suppression observed on days 5 and 6 in cultures to which Con A-activated suppressor T cells were added at initiation was not due to a failure to initiate the PFC response. On days 2 and 3 of culture, PFC responses in cultures containing suppressor cells were the same or slightly greater than responses in cultures to which non-Con A-activated control spleen cells were added. The PFC responses in cultures containing the suppressor cells aborted dramatically, however, after 72 hrs, at the time when the PFC responses in control cultures was expanding exponentially. From days 4 to 6 when PFC responses in control cultures were maximum, PFC responses in cultures containing suppressor cells fell precipitously (51). A similar pattern of suppression was observed in CL responses generated in MLC; on days 3 and 4, CL responses in control cultures and cultures to which suppressor

cells in vitro were profoundly suppressed by mitogenic concentrations (1 µg/ml) cells had been added at initiation were similar. On days 4 through 6, CL responses in control cultures increased progressively, however, during this interval, a definite and continued inhibition of CL responses was observed in cultures with suppressor cells (49). The dichotomy between the time early in the response when suppressor T cells must be present to achieve suppression and the later time during expression of maximum responses in control cultures when suppression of PFC and CL responses was actually manifested led to experiments investigating the mechanisms involved. a population of suppressor T cells.

In these experiments, we found that supernatant fluids from cultures of Con A-activated spleen cells, from which residual Con A had been removed by Sephadex absorption, contained factors, termed soluble immune response suppressors (SIRS), which suppressed PFC responses without cytotoxicity on responding spleen cells. Kinetic analysis of PFC responses revealed a pattern of suppression identical to that observed with suppressor cells (51). However, to date we have been unable to suppress CL responses with SIRS (30, 31, 60); the reasons for this failure require further experimentation which may provide additional information about the mechanisms of action of Con A-activated suppressor T cells and SIRS.

SIRS activity was detected in supernatant fluids of Con A-activated spleen cells within 6 hrs, and was maximal in fluids harvested between 12 and 48 hrs after initiation (51). Interestingly, the kinetics of secretion of SIRS is similar to that of mitogen-induced secretion of the T cell mediators MIF (61) and interferon (62). Supernatant fluids with SIRS activity also have MIF activity; in all of the analyses to date we have been unable to dissociate definitively SIRS and MIF activity (31, 63). Physicochemical characterization has shown that SIRS is a heterogeneous glycoprotein with a molecular weight in the range of 35,000 to 68,000 daltons which is soluble in 70% $(NH_4)_2SO_4$ and stable at 56°C for 60 min, but destroyed in 10 min at 70°C and at pH 2. SIRS is not absorbed by antigen, mouse immunoglobulins, or antisera against mouse immunoglobulins or histocompatibility antigens, but is absorbed by spleen cells from several mouse strains. SIRS also has no strain specificity in its effects (31, 63). Further, SIRS mediates its suppression by acting on macrophages and not T cells or B cells, and, like MIF, can be blocked by L-fucose, adding further support to the notion that SIRS and MIF may be the same molecules (31, 64).

Suppressor T cells and their products generated by activation of T cells with Con A provide a viable experimental system to probe the biology of suppressor T cells. Many experiments are needed to characterize these suppressor T cells and SIRS more precisely and to determine their mechanism(s) of action in suppression of PFC responses, and why SIRS fails to suppress CL responses. These experiments will be described in Section 3.

However, Con A-induced suppressor T cells and SIRS have the disadvantage of being non-antigen specific in their effects. Preliminary observations from our laboratory indicate that antigen-specific suppressor T cells can be generated in tissue culture in certain circumstances. First, T cells harvested from MLC on days 6 or 7, after the peak of a CL response, specifically suppress generation of CL responses to the same alloantigens by normal syngeneic cells. Second, T cells harvested from a culture after the peak of a PFC response specifically suppress

PFC responses to the same antigen by normal spleen cells. The detailed investigation of these preliminary observations is the basis for part of this application.

II. Specific Aims. The specific aims of this project have been divided into two groups:

A. Further characterization of Con A-activated suppressor T cells and their products (SIRS)

1. Investigation of the metabolic activities required for activation of suppressor T cells, secretion of SIRS, and expression of biological activity by these T cells.
2. Further physicochemical characterization of SIRS and comparison with other T cell mediators.
3. Investigation of the effects of SIRS on the functions of macrophages and evaluation of secondary effects of SIRS-activated macrophages on the functions of T cells and B cells.
4. Investigation of the reasons for the failure of SIRS to suppress CL responses.

B. Development of experimental systems for generation of antigen-specific suppressor T cells and their products

1. Definition of parameters for generation of antigen-specific suppressor T cells and their soluble products.
2. Physicochemical characterization of active T cell products and comparison with Con A-induced mediators.
3. Determination of the cellular site(s) of action and mechanism(s) by which these T cells and their products suppress immune responses.
4. Development of an experimental model which allows antigen-specific suppression of antibody responses without affecting cell-mediated responses to the same antigen and vice versa.

III. Methods of Procedure. The experimental systems necessary for investigating the mechanisms by which suppressor T cells and their products regulate immune responses are routine procedures in this laboratory. Initially tissue culture systems will be used exclusively, but in later stages of the project *in vivo* animal models will be used.

Culture and Assay Systems. Antibody responses will be generated in cultures of mouse spleen cells using the system of Mishell and Dutton (65) as modified in our laboratory (66). Stimulating antigens will be sheep erythrocytes (SRBC), the synthetic polypeptide antigen GAT, and mouse alloantigens coupled to pigeon erythrocytes. IgM and IgG PFC responses will be measured by the hemolytic plaque technique using SRBC or the determinant of interest coupled to SRBC (66, 67). Cytotoxic lymphocytes (CL) responses will be generated by incubating responder spleen cells with appropriate mitomycin C-treated allogeneic spleen cells in Mishell-Dutton type cultures. CL responses will be measured in the standard ^{51}Cr release assay using P815 mastocytoma or EL-4 leukemia as target cells (49). A recently developed system which allows the generation of simultaneous and non-cross-reactive CL responses to two sets of alloantigens in MLC (68) will be used in the studies of antigen-specific suppressor T cells and their products.

Generation of Suppressor T Cells and SIRS by Con A. Suppressor T cells will be generated by incubating spleen cells with Con A (1 µg/ml) for 48 hrs. After

separations of the cells, supernatant fluids will be absorbed with Sephadex G75 to remove residual Con A; the cells will be washed three times with medium. Control spleen cells will be incubated for 48 hrs without Con A, which will be added at the time of harvest of the cultures; thereafter, these cells and supernatant fluids will be washed or absorbed as described for the Con A-activated preparations (31, 48, 49, 51, 63). Since SIRS is soluble in 70% $(NH_4)_2SO_4$, crude supernatant fluids will be purified initially by precipitation of irrelevant material by 70% $(NH_4)_2SO_4$. Further treatment of the cells, purification procedures for SIRS, and the preparation, testing, and purification of antigen-specific suppressor T cells and their products will be described in later sections. [illegible] SIRS, and expression of biological activity by these T cells.

Specific Experimental Protocols. [illegible]

A. Further characterization of Con A-activated suppressor T cells and their products (SIRS).

[illegible] on the function of T cells and B cells.

1. Investigation of the metabolic activities required for the activation of suppressor T cells, secretion of SIRS, and expression of biological activity by these T cells. Previous studies have shown that X-irradiation (2000 R) of spleen cells prior to activation with Con A abrogates generation of suppressor T cells, but after activation, suppressor T cell function is radioresistant (48, 49). Further experiments will determine whether X-irradiated T cells stimulated with Con A can secrete SIRS (tested on PFC responses). In addition, the effects of inhibitors of DNA synthesis (hydroxyurea or mitomycin C), RNA synthesis (chromomycin A_3 or actinomycin D) and protein synthesis (cycloheximide or puromycin) on generation of Con A-activated suppressor T cells (tested on PFC and CL responses) and secretion of SIRS (tested on PFC responses) will be determined. It is anticipated that inhibitors of protein synthesis and probably RNA synthesis will block production of SIRS and that inhibitors of DNA synthesis will block generation of suppressor T cells. These same inhibitors, plus colchicine and cytochalasin B (to disrupt microtubules) and antimycin A (to inhibit oxidative phosphorylation) will be used to determine which metabolic activities are required for expression of the biological activity of suppressor T cells (tested on PFC and CL responses). Inhibitors of DNA synthesis are expected to be without effect in this regard. Other metabolic inhibitors will be used if indicated by these studies. This analysis will be useful in determining whether suppressor T cells mediate their effects on immune responses only by secretion of SIRS or whether some other activities mediated directly by the cells are responsible. This determination is crucial in analyzing why SIRS does not suppress CL responses (See Section A.4 below).

[illegible] synthetic polypeptide antigen GAT, and mouse alloantigens could be given

2. Physicochemical characterization of SIRS and comparison with other T cell mediators. Supernatant fluids from cultures of Con A-activated spleen cells have SIRS and MIF activity. In all of the separatory and analytical procedures used, including column chromotography on Sephadex G100, polyacrylamide gel electrophoresis, CsCl density gradient ultracentrifugation, treatment with proteolytic enzymes and absorption with a variety of insoluble immunologically relevant materials, we have been unable to dissociate MIF and SIRS activity (31, 63). In addition, these supernatant fluids should have interferon activity (62), another T cell mediator which inhibits PFC responses *in vitro* in a manner analogous to SIRS (69). However, SIRS is labile at pH 2, suggesting it is not interferon Type I (69). Younger and Salvin (70) have described a Type II interferon which is

also labile at pH 2 but otherwise indistinguishable from mouse MIF. Thus, SIRS will be analyzed for interferon activity by standard methods (71); we expect to find interferon activity in these preparations. After each future purification or treatment procedure, the preparations will be analyzed for SIRS, MIF, and interferon activity in an attempt to distinguish among these mediators. Dr. Salvin has agreed to exchange reagents with us in an attempt to resolve this issue.

Further purification and characterization procedures will be aided by internally labeling SIRS with ^{14}C amino acids and by precipitation of irrelevant material with 70% $(NH_4)_2SO_4$. Since SIRS is a glycoprotein, based on CsCl density gradient ultracentrifugation (31, 63), it will be purified further by passage over a Con A-Sepharose column; non-bound and methylmannoside elutable material will be assayed for biological activity. Further characterization will involve determining the sensitivity of the various biological activities to the action of several glycosidases, including neuraminidase, L-fucosidase, B-galactosidase, and N-acetylglucosaminidase (72). This analysis will identify which, if any, of the sugar moieties in the glycoprotein are necessary for its biological activities.

L-fucose partially inhibits the ability of SIRS to suppress PFC responses (30, 64). The capacity of various other sugars (72), including sialic acid and several hexoses, hexosamines, and N-acetylhexoamines, to competitively inhibit or absorb SIRS, MIF, and interferon activity will be investigated. In absorption studies, SIRS will be passed over Sepharose columns to which the sugar moieties have been coupled in an insoluble form and the effluent will be assayed for biological activity. For competitive inhibition studies with the sugars, advantage will be taken of the fact that SIRS acts on macrophages and that exposure of macrophages to SIRS for 2 hrs is sufficient to achieve suppression of the PFC responses generated by T and B lymphocytes added to the treated macrophages (31, 64). Thus, separated macrophages can be treated with SIRS in the presence of the various sugars and washed before addition of lymphocytes, thus avoiding the non-specific toxic effects of some of the sugars on lymphocytes. Modifications of this approach will be used in assays for MIF and interferon activity. This analysis will determine which sugars, in addition to L-fucose, are involved in the binding of SIRS to macrophages and other biological activities of SIRS, e.g. MIF and interferon activity.

3. Effects of SIRS on functions of macrophages and evaluation of secondary effects of SIRS-activated macrophages on T cells and B cells. SIRS acts on macrophages, but does not block initiation of the PFC response (31, 64). Instead, after developing normally during the first 3 days of culture, PFC responses abruptly abort and the number of PFC decrease precipitiously on days 4 through 6 (30, 31, 51). Furthermore, macrophages treated with SIRS have no obvious defects in uptake, catabolism, or retention of the soluble antigen, GAT (64). SIRS may interfere with functions of macrophages essential later in the PFC response, or SIRS may activate macrophages so that they or their products are actively inhibitory. Since macrophages are not required in the cultures after 48 hrs incubation for development of normal PFC responses (73), the latter alternative seems more likely at this time. Initially, the effects of SIRS on the morphology and accessory functions of macrophages will be evaluated. Morphological changes, such as increased spreading of cytoplasmic processes on the culture dish, "ruffling" of the plasma membrane and increased numbers of cytoplasmic lysosomes, all of which are characteristic of "activated macrophages" (25, 26, 72) are expected and will be surveyed

also labile at pH 2 but otherwise indistinguishable from mouse MIF. The SIRS with phase or scanning electron microscopy. The loss of membrane proteoglycan residues, which is characteristic of MIF-activated macrophages, will be evaluated by staining SIRS-treated and untreated macrophages with ferrocyanide reduced osmium tetroxide (74). Functions characteristic of activated macrophages, such as increased phagocytic capability, cytotoxicity or cytostasis of tumor cells, and increased bactericidal activity (25, 26, 72, 75) will be evaluated. This analysis will provide information on the effects of SIRS on other physiologic functions of macrophages. [illegible] amino acids and by precipitation of irrelevant material with [illegible]. Since SIRS is a glycoprotein based on [illegible] grad[illegible] The possibility that SIRS-treated macrophages elaborate low molecular weight products, similar to those shown in other systems to inhibit lymphocyte or tumor cell proliferation (75) will be determined, as well as their ability to secrete collagenase (77). If these macrophages elaborate inhibitory low molecular weight products, the products, their target cells, and mechanisms of action will be determined. If these macrophages elaborate collagenase, the effects of this enzyme on PFC responses and its relevance to SIRS-induced inhibition of PFC responses will be determined. In conjunction with the studies on production of factors by macrophages, the rates of DNA, RNA, and protein synthesis at specific intervals in suppressed cultures, as determined by incorporation of ^{3}H-thymidine, ^{3}H-uridine, and ^{3}H-leucine, respectively, will be correlated with the abortion of the PFC response after day 3 of culture. This analysis will reveal whether the abortion of the PFC response is due to cessation of proliferation of precursors of PFC, or cessation of antibody synthesis by these cells which continue to proliferate at a normal rate.

Treatment of macrophages with proteolytic enzymes (trypsin or chymotrypsin) and various glycosidases (fucosidase, neuraminidase, and N-acetylglucosaminidase) (72) before reaction with SIRS should provide information as to what moieties SIRS interacts with on macrophage membranes. The effects of these enzymes on the function of macrophages in PFC responses will be determined for control purposes. These results will be correlated with the results of experiments described in Section A.2 and should provide information about the receptor sites on both macrophages and SIRS which are critical for SIRS-induced suppression of PFC responses. Further, membrane esterases inhibit the effect of MIF on macrophages (72). Working on the assumption that MIF and SIRS activity are not dissociable, the effects of inhibiting this esterase activity by diisoflurophosphate or α_1 anti-trypsin (25, 26, 72) on the SIRS-induced inhibition of PFC responses will be determined. This analysis will be particularly useful when applied to peritoneal exudate macrophages, which are much less susceptible to the effects of SIRS than splenic macrophages (64). It is conceivable that peritoneal macrophages have higher levels of membrane esterase activity than splenic macrophages and that this accounts for the inability of SIRS to mediate its effect on peritoneal macrophages. It is anticipated that peritoneal macrophages will be susceptible to effects of SIRS after inhibition of membrane esterases.

4. Analysis of why SIRS fails to suppress CL responses. These experiments may be unnecessary if the experiments described in Section A.1 show that suppressor T cells regulate immune responses by mechanisms other than the secretion of SIRS.

If this series of experiments is necessary, the effects of SIRS on T cell proliferative responses to Con A, PHA, and allogeneic cells measured by ^{3}H-thymidine

incorporation will be determined to evaluate the ability of SIRS to suppress other T cell responses.

In the MLC, biologically active products are elaborated which enhance immune responses (45, 78); these products may override the suppressive effects of SIRS in the generation of CL responses. To test this possibility, soluble products from an MLC will be added in varying concentrations with SIRS to cultures of spleen cells stimulated with SRBC to determine whether the SIRS-mediated suppression of the PFC response is overriden by MLC products. This indirect test should provide evidence as to whether a similar mechanism accounts for failure of SIRS to inhibit CL responses.

Lastly, the larger numbers of macrophages in cultures used to generate CL responses may circumvent SIRS-mediated suppression. Addition of an excess of macrophages to SIRS-treated cultures often overcomes suppression of PFC responses (64). Thus, the number of macrophages in cultures used to generate CL responses will be reduced as much as possible without compromising the CL responses and the effects of SIRS in these cultures will be evaluated.

However, from our experiences with these experimental systems, it is our expectation that SIRS will be unable to suppress CL responses, and that these responses will be suppressed by Con A-activated suppressor T cells by mechanisms other than the activity of SIRS. These mechanisms will be probed first by determining the target cell(s) (macrophages, T cells, or B cells) of the suppressor T cells and then by determining the specific effects of these cells on the target cell(s). If this analysis is necessary, we anticipate that it will be one of the most difficult portions of the project.

B. Development of experimental systems for generation of antigen-specific suppressor T cells and their products.

1. Definition of parameters for generation of antigen-specific suppressor T cells and their soluble products. This portion of the project is crucial to the objective of developing experimental systems which allow selective suppression of antibody responses without affecting cell-mediated immune responses to the same antigen and vice versa. Our observations with the generation of antigen-specific suppressor T cells and their products are preliminary; because of our relative ignorance about these systems, this portion of the application will, of necessity, be less specific and more tentative. However, we intend to take full advantage of lessons learned in studies with Con A-induced suppressor T cells and SIRS and apply them in this portion of the project. The observations that antigen-specific suppressor T cells can be recovered from cultures after the peak of either a PFC response to SRBC or a CL response to allogeneic cells provide the starting point for this project.

The culture conditions for recovery of optimal suppressor T cell activity after the peak of a PFC or CL response will be determined initially. The T cells will be purified on appropriate immunoadsorbent columns (36, 79), identified as T cells by sensitivity to anti-θ serum and complement, and tested for their ability to specifically suppress PFC or CL responses to the antigens which stimulated their generation. These T cells will also be incubated with specific or irrelevant antigen to induce production of biologically active soluble products. If such products cannot be recovered in culture supernatant fluids, the cells will be

sonicated to release these products (80), which, after ultracentrifugation to remove cell debris, will be tested for specific suppressive activity on PFC and CL responses. The rationale for this approach is derived from the observations that suppressor T cells or their products normally act physiologically to limit the duration and/or magnitude of immune responses, i.e. this is a normal physiological homeostatic mechanism (29-31).

Another approach will be to incubate suspensions of T cells (thymocytes, cortisone-resistant thymocytes, or purified peripheral T cells) with appropriate antigen for various periods of time, either with or without macrophages. The cells and supernatant fluids (after removal of any residual antigen by immunoabsorbent columns) will be tested for specific suppressive activity on PFC and CL responses.

These experiments will provide useful information about the generation and biology of antigen-specific suppressor T cells and their biologically active products in general. However, one major thrust of this project is to generate suppressor T cells and/or their products which will specifically suppress antibody responses to mouse alloantigens without compromising cell-mediated immune responses to cells bearing the same alloantigens. Thus, alloantigens in membranes of lymphocytes from appropriate mouse strains will be internally labeled with ^{35}S methionine or trace labeled with ^{125}I using the lactoperoxidase method (81) and extracted with NP40 or other suitable detergents (82). The desired alloantigens, products of the H-2 gene complex, will be purified on appropriate immunoadsorbent columns of insolubilized anti-H-2 sera (83). CL responses to these alloantigens will be generated by incubating responder spleen cells with appropriate mitomycin C-treated allogeneic spleen cells in the usual fashion. Soluble alloantigens will be coupled to pigeon erythrocytes with carbodiimide (84) for stimulation of PFC responses in Mishell-Dutton cultures. PFC responses will be measured in the hemolytic plaque assay using the alloantigens coupled to SRBC as indicator cells. The development of this system will permit determination of the critical parameters for specific suppression of PFC responses without affecting CL responses to the same antigens using the antigen-specific suppressor T cells and their products generated as described above. The system for generating simultaneous non-cross-reactive CL responses to two sets of alloantigens (68) will be especially useful in this portion of the project.

2. Physicochemical characterization of active T cell products.

Antigen-specific suppressor T cell products will be purified and characterized by the same battery of techniques which have been used with SIRS. Sephadex G100 and G200 column chromatography, polyacrylamide gel electrophoresis including SDS and isoelectric focusing gels (85), and CsCl density gradient ultracentrifugation will be used to determine molecular weight, electrophoretic mobility, relative carbohydrate content, and molecular chain composition. Sensitivity to temperature, proteolytic enzymes, glycosidases, and nucleases will also be determined to further characterize the active molecules. The nature of the receptors on these molecules for cells or antigen will be evaluated by determining the ability of macrophages, T cells and B cells and specifically reactive columns (83) to absorb the biological activity. Alloantigen, mouse immunoglobulin, various sugars, Con A, anti-immunoglobulin, and anti-histocompatibility antigen columns will be used initially. Other specifically reactive columns will be used as dictated by the results of these studies. These T cell products will also be evaluated for MIF and interferon activity. Further purification and characterization will be employed as needed. After each procedure, the preparation will be tested for biological activity on PFC and CL responses to mouse alloantigens. The characterization of these molecules is

a critical first step in understanding the mechanisms of their biological activity. It is anticipated that these products may be identified as products of the major histocompatibility or H-2 gene complex (33, 43, 45, 53).

3. Determination of cellular site(s) and mechanism(s) of action of antigen-specific suppressor T cells and their products. This portion of the project will be greatly simplified if we have succeeded in obtaining an antigen-specific T cell factor which suppresses antibody responses, but not CL responses, and the reverse. In this case, the minimum exposure time of responding spleen cells to the factor required to suppress PFC responses, for example, to a given antigen, will be determined. Then purified macrophages, T cells and B cells will be exposed to the factor and cultures containing all the possible combinations of treated and normal cells will be evaluated to determine the target cells of the factor. A similar approach will be used to determine the target cell for suppression of CL responses. Depending on the results of these analyses, the effects on functions which the target cell usually performs in the immune response will be analyzed. For example, the effects on a) antigen presentation functions of macrophages, b) cooperative interactions of T cells with B cells, and, c) actual antibody production by B cells will be determined. The determination of the effects of suppressor T cells or their products on the kinetics of development of PFC and CL responses will be especially important. Failure to initiate a response suggests an effect on macrophages, whereas suppression of the responses with a pattern similar to that observed with SIRS would indicate that some event after successful initiation of the response has been affected. PFC and CL responses and DNA, RNA, and protein synthesis in suppressed cultures will also be correlated to determine the mechanism of action of the factor. Further analyses will be carried out depending on the results of these experiments.

If suppressor T cells are required to achieve the desired effects, a similar pattern of attack will be employed, but the analysis will be considerably more complicated, as indicated above for the site(s) and mechanism(s) of action of Con A-activated suppressor T cells.

4. Development of the experimental model. If this project has developed successfully using the approaches outlined above, we will be able to specifically suppress antibody responses to an antigen without affecting cell-mediated immune responses to the same antigens and vice versa. This experimental system will be developed and defined using tissue culture systems. Once operational, the experimental system will be used to study regulation of antibody and cytotoxic lymphocytes responses *in vivo*. Eventually, this system could be used to probe regulatory mechanisms in response to tumor-specific antigens.

IV. Significance

The significance of various experimental procedures and anticipated results has been pointed out in the descriptions of methods of procedure.

The understanding of mechanisms which regulate development and expression of humoral and cell-mediated immune responses is crucial for evaluating the effects of environmental factors, such as cigarette smoking, on the cells and responses of the immune mechanism. Further, the understanding of these regulatory mechanisms should permit manipulation of the immune mechanism for alleviation of disease processes with immunological components.

This project is designed in part to investigate in detail the mechanisms by which mitogen-induced, non-antigen-specific suppressor T cells and their secreted mediators (SIRS, MIF, ?interferon) regulate development and expression of humoral and cell-mediated immune responses. The information derived from these studies should contribute significantly to our understanding of normal physiological regulatory mechanisms in immune responses, since both MIF and interferon are physiological mediators which may normally influence development and expression of immune responses.

The development of experimental systems to selectively suppress specific humoral immune responses has direct relevance to control of neoplastic processes, some of which are thought to have cigarette smoking as an etiological component. Antibody responses to tumor antigens often interfere with successful development and expression of cell-mediated immune responses to tumor cells (86, 87). Since a functional cell-mediated immune response is crucial for destruction of the tumor cells, experimental manipulations which eliminate antibody responses to the tumor without compromising cell-mediated immune responses will be especially valuable. It is also highly desirable to suppress antibody responses to only specific antigens, since non-specific suppression of all antibody responses lays the host open to a myriad of other problems. The second portion of this application is directed to this objective.

V. Time Required for This Project

The portion of the project dealing with the characterization and definition of mechanisms of action of Con A-activated suppressor T cells and SIRS will require approximately two years to complete. The portion of the project dealing with generation and characterization of antigen-specific suppressor T cells and their products will require two to three years to complete depending on the rates of success in the initial experiments.

VI. Human Subjects

No human subjects or materials from humans will be used in the proposed studies.

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10. Space and facilities available (when elsewhere than item 2 indicates, state location):

The laboratory facilities are in the Department of Pathology at Harvard Medical School. The research space available for this project is 960 sq. ft. subdivided as follows: semi-isolated tissue culture area, 150 sq. ft.; adjacent walk-in 37°C warm room, 150 sq. ft.; general laboratory area, 570 sq. ft.; and office, 90 sq. ft. Major items of permanent equipment include: refrigerated centrifuges with accessories; microscopes with phase optics; inverted tissue culture phase microscope with photographic accessories; Coulter Counter with size-distribution plotter; Cytograf 6300A for viable cell counting; water jacketed CO_2 incubator; laminar flow tissue culture hoods; pH meter; semi-microbalance; serological water baths; tissue culture incubation chambers; rocker platforms; and refrigerator-freezer.

The Department of Pathology provides the following in support of this project: animal housing facilities; cold rooms; dark rooms; G.E. Maximar 250-III X-irradiation facility; glassware washing and preparation facilities; and tissue processing and histology facilities. The Department also provides the following items of major equipment: spectrophotometers; high speed and ultracentrifuges with rotors; Revco -70°C freezers; gamma and beta scintillation spectrometers; apparatus for programmed freezing of cell suspensions; flourescence, transmission, and scanning electron microscopes and photomicrography equipment; electronic calculating machines and photocopying facilities.

Drs. John David and Heinz Remold of the Robert B. Brigham Hospital will continue to be consultants and active collaborators in aspects of this project involving the characterization of non-specific and antigen-specific suppressor T cell products. Dr. Alice Huang of the Department of Microbiology and Molecular Genetics will be a consultant in aspects of this project involving interferon assays.

11. Additional facilities required: None

12. Biographical sketches of investigator(s) and other professional personnel (append):

13. Publications: (five most recent and pertinent of investigator(s); append list, and provide reprints if available).

12. Biographical Information

Carl William Pierce

Born: REDACTED

Education:

REDACTED A.B., cum laude, Colgate University, Hamilton, New York
M.D., with honors, University of Chicago, Chicago, Illinois
Ph.D., University of Chicago (Pathology), Chicago, Illinois

Research and Professional Experience:

1962 - 1963	U.S.P.H.S. Predoctoral Trainee in Pathology, University
1964 - 1965	of Chicago, Chicago, Illinois
1966 - 1967	Intern and Assistant in Pathology, University of Colorado, Denver, Colorado
1967 - 1970	Research Associate, Laboratory of Immunology, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland
1970 - 1973	Assistant Professor of Pathology, Harvard Medical School, Boston, Massachusetts
1973 -	Associate Professor of Pathology, Harvard Medical School, Boston, Massachusetts

Memberships in Professional Societies:

1963
1964
1967
1967 REDACTED REDACTED
1970
1970

Honors and Awards:

1972 - 1977	Recipient of Research Career Development Award from National Institute of Allergy and Infectious Diseases, N.I.H., U.S.P.H.S.

Editorial Boards and Other Appointments:

1972 - 1974	Program Committee, American Association of Immunologists
1974 -	Associate Editor, The Journal of Immunology

Research Interests:

Immunobiology

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13. Pertinent Recent Publications

Carl William Pierce

1. Pierce, C.W., T. Tadakuma, A.L. Kühner, and J.R. David, Characterization of a soluble immune response suppressor (SIRS) produced by concanavalin A-activated spleen cells. *In:* The Role of Mitogens in Immunobiology. J.J. Oppenheim, D.L. Rosenstreich, and M. Landy (Eds.), Academic Press, N.Y., in press.

2. Pierce, C.W., D.L. Peavy, and T. Tadakuma, Suppressor T cells as regulators of lymphocyte functions. Ann. N.Y. Acad. Sci., in press.

3. Pierce, C.W., J.A. Kapp, S.M. Solliday, M.E. Dorf, and B. Benacerraf, Immune responses *in vitro* XI. Suppression of primary IgM and IgG plaque-forming cell responses *in vitro* by alloantisera against leukocyte alloantigens. J. Exp. Med. *140:*921, 1974.

4. Kapp, J.A., C.W. Pierce, S.F. Schlossman, and B. Benacerraf, Genetic control of immune responses *in vitro*. V. Stimulation of suppressor T cells in nonresponder mice by the terpolymer L-glutamic acid^{60}-L-alanine^{30}-L-tyrosine^{10} (GAT). J. Exp. Med. *140:*648, 1974.

5. Peavy, D.L. and C.W. Pierce, Cell-mediated immune responses *in vitro*. I. Suppression of the generation of cytotoxic lymphocytes by concanavalin A and concanavalin A-activated spleen cells. J. Exp. Med. *140:*356, 1974.

12. Biographical Information

Takushi Tadakuma

Born: REDACTED

Nationality:

Education:

Premedical Course, Keio University, Tokyo, Japan
M.D., Keio University School of Medicine, Tokyo, Japan
D.M.S., Keio University Graduate School of Medicine (Microbiology and Molecular Biology), Tokyo, Japan

Research and Professional Experience:

1965 - 1966	Intern, Keio University Hospital, Tokyo, Japan
1966 - 1970	Fellow, Keio University Graduate School of Medicine, Tokyo, Japan
1970 - 1973	Instructor, Department of Microbiology, Keio University, Tokyo, Japan
1973 - Present	Assistant Professor, Department of Microbiology, Keio University, Tokyo, Japan (on leave of absence)
1973 - 1975	Research Fellow, Department of Pathology, Harvard Medical School, Boston, Massachusetts
1975 -	Instructor, Department of Pathology, Harvard Medical School, Boston, Massachusetts

Memberships in Professional Societies:

1967
1967
1971

REDACTED

REDACTED

Research Interests:

Immunobiology

13. Pertinent Recent Publications

1. Pierce, C.W., T. Tadakuma, A.L. Kühner, and J.R. David, Characterization of a soluble immune response suppressor (SIRS) produced by concanavalin A-activated spleen cells. *In:* The Role of Mitogens in Immunobiology. J.J. Oppenheim, D.L. Rosenstreich, and M. Landy (Eds.), Academic Press, N.Y., in press.

2. Pierce, C.W., D.L. Peavy, and T. Tadakuma, Suppressor T cells as regulators of lymphocyte functions. Ann. N.Y. Acad. Sci., in press.

12. Biographical Information

3. Tadakuma, T., T. Mitsuma, *et al.*, *In vitro* reconstitution of anti-sheep erythrocyte antibody response of T cell-depleted spleen cells by allogeneic T cells or factors derived from them. Japan J. Microbiol., in press.

4. Tadakuma, T., Cell cooperation in anti-sheep red blood cell antibody responses in mouse spleen cell cultures: Use of anti-lymphocyte globulin for selective suppression of the antigen reactive cells. Japan J. Microbiol., *16*:287, 1972.

5. Tadakuma, T., K. Saito, *et al.*, Initiation of the primary immune response to sheep red blood cells in the dissociated mouse spleen cell culture. II. Histochemical study on the cell clusters developed during the *in vitro* immune response. Japan J. Microbiol., *15*:493, 1971.

13. Pertinent Recent Publications

12. Biographical Information

Duane Lee Peavy

Born: REDACTED

Education:

REDACTED B.S., with distinction, Ohio State University, Columbus, Ohio
Ph.D., University of Florida (Immunology and Medical Microbiology), Gainesville, Florida

Research and Professional Experience:

1968 - 1972	U.S.P.H.S. Predoctoral Trainee in Immunology and Medical Microbiology, University of Florida, Gainesville, Florida
1973 -	Research Fellow, Department of Pathology, Harvard Medical School, Boston, Massachusetts

Memberships in Professional Societies:

1972
1973 REDACTED REDACTED
1973
1975

Honors and Awards:

1968	Graduated with distinction in Microbiology, Ohio State University
1972	Sigma Xi Graduate Student of the Year, University of Florida
1974 - 1976	Recipient of Postdoctoral Fellowship from the National Cancer Institute, N.I.H., U.S.P.H.S.

Research Interests:

Immunobiology

13. Pertinent Recent Publications

1. Peavy, D.L. and C.W. Pierce. Cell-mediated immune responses *in vitro*. III. Elimination of specific cytotoxic lymphocyte responses by ^{3}H-thymidine suicide. Submitted to J. Immunol, 1975.

2. Peavy, D.L. and C.W. Pierce. Cell-mediated immune responses *in vitro*. II. Simultaneous generation of cytotoxic lymphocytes to two sets of alloantigens of limited cross-reactivity. Submitted to J. Immunol, 1975.

3. Pierce, C.W., D.L. Peavy, and T. Tadakuma. Suppressor T cells as regulators of lymphocyte functions. Ann. N.Y. Acad. Sci., in press.

Duane Lee Peavy

4. Peavy, D.L. and C.W. Pierce, Cell-mediated immune responses *in vitro*. I. Suppression of the generation of cytotoxic lymphocytes by concanavalin A and concanavalin A-activated spleen cells. J. Exp. Med.,*140*:356, 1974.

5. Peavy, D.L., W.H. Adler, J.W. Shands, and R.T. Smith, Mitogenic effect of endotoxin on mouse lymphoid cells: Thymus independence of LPS. Cell. Immunol., *11*:86, 1974.

14. First year budget:

A. Salaries (give names or state "to be recruited") Professional (give % time of investigator(s) even if no salary requested)	% time	Amount Salary	Fringe Benefits	Total
Carl W. Pierce, Principal Investigator	30			
Takushi Tadakuma, Instructor	100			
Duane L. Peavy, Instructor	100	REDACTED		
Technical				
Research Assistant I, to be recruited	100			
Deborah Siner, Editorial Assistant and Secretary	25	REDACTED		
		REDACTED		
Sub-Total for A				

B. Consumable supplies (by major categories)	
Animals and animal care supplies	3,500
Tissue culture supplies	3,500
Reagents, sera, radioisotopes	2,500
Glassware and general supplies	500
Sub-Total for B	$10,000

C. Other expenses (itemize)	
Domestic travel	500
Portion of service contracts on major equipment shared with other members of Department	300
Publication costs (art work, page costs, reprints)	200
Sub-Total for C	$ 1,000
Running Total of A + B + C	$39,425

D. Permanent equipment (itemize)

None

Sub-Total for D	$ 0
E	$ 5,914
Total request	$45,339

E. Indirect costs (15% of A+B+C)

15. Estimated future requirements:

	Salaries	Consumable Suppl.	Other Expenses	Permanent Equip.	Indirect Costs	Total
Year 2		10,750	1,075	0	7,575	$53,078
Year 3	REDACTED	1,556	1,156	0	8,144	$62,435

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Budget Explanations and Justifications

A. Rationale for this application

This research project has been developed both from the results of other investigators and from experiments in my laboratory currently supported by grants from the National Institutes of Health (see item 16). Insufficient funds are available in these grants to pursue the proposed project in the desired fashion without compromising the progress of other important projects. Further, since some of the objectives of the proposed project are outside the major objectives of these grants, it is inappropriate to continue to support the proposed project entirely with funds from these grants. Our experience with the experimental approaches and methodology to be used have demonstrated that this project is feasible and will provide significant new and relevant information about the mechanisms by which suppressor T cells and their products regulate humoral and cell-mediated immune responses.

B. Personnel

This project, as conceived, will require the full-time efforts of the two Instructors, who are currently actively involved in various aspects of the project, and a Research Assistant. The Principal Investigator will devote at least 30% of his time to the supervision of the progress of the project and actual experimentation. Dr. Tadakuma, Instructor, is on leave of absence from Keio University Medical School, Tokyo, Japan, which provides approximately half his salary. Funds are requested for the remainder of his salary and fringe benefits (16%, on that portion of salary requested only) to bring his total remuneration to a level commensurate with other Instructors with comparable training and experience. Dr. Duane L. Peavy will be supported by an N.I.H. Postdoctoral Fellowship until June 30, 1976, at which time he will be appointed Instructor. Funds for salary and fringe benefits (16%) from July 1 to December 31, 1976 are requested. The increased salary budget in years 2 and 3 reflects, in part, Dr. Peavy's salary on a 12-month basis. The continuation of these two Instructors, who are presently involved with aspects of this project, will guarantee uninterrupted progress of experimentation. Since this grant, if funded, will support approximately 25% of the research activities of the laboratory, funds are requested for 25% of the salary and fringe benefits (14.5%) of Ms. D. Siner, Editorial Assistant and Secretary, who is presently supported entirely by the listed N.I.H. Grants. Fringe benefits for the Research Assistant are 14.5%. An annual salary increase of 7.5% for personnel is requested.

C. Consumable Supplies

Funds requested in each category are estimated from current operating expenses for those portions of the project already in progress and are a realistic estimate of the funds necessary to carry out the proposed project in the desired fashion. An annual increase of 7.5% for supplies is requested.

D. Other Expenses

Travel funds to enable Drs. Tadakuma and Peavy to attend one scientific

meeting per year are requested. Funds for a portion of service contracts on major equipment shared with other members of the Department are requested. Based on our experience with the proposed projects, publications in the first year are almost assured, and therefore funds for publication costs (page charges, art work, and 200 reprints) are also requested. An annual increase of 7.5% in this category is requested.

16. Other sources of financial support:
List financial support from all sources, including own institution, for this and related research projects.

CURRENTLY ACTIVE

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates
1. Cell Interactions in Immune Responses *in Vitro*	National Institutes of Health Research Grant AI-09897	$204,050	9/1/73 - 8/31/78
		$ 40,000	9/1/75 - 8/31/76
2. Studies on Experimental Cancer Immunology, Project V: Studies on Tumor Specific Immunity *in Vitro*.	National Institutes of Health Program Project Grant CA-14723	$134,744	6/30/73 - 5/31/78
		$ 26,177	6/1/75 - 5/31/76

PENDING OR PLANNED

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates
Biology of Suppressor T Cells	American Cancer Society (pending)	$ 86,373	1/1/76 - 12/31/77
		$ 42,509	1/1/76 - 12/31/76

(The application to the A.C.S. is similar, but not identical, to the present application. If both applications should be funded, only one award would be accepted.)

It is understood that the investigator and institutional officers in applying for a grant have read and accept the Council's "Statement of Policy Containing Conditions and Terms Under Which Project Grants Are Made."

Principal investigator

Typed Name Carl W. Pierce

Signature Carl W. Pierce Date 6/25/75

Telephone 617-734-3300, Extension 337
Area Code Number Extension

Checks payable to
Harvard Medical School, c/o Business Office

Mailing address for checks
25 Shattuck Street
Boston, Massachusetts 02115

Responsible officer of institution

Typed Name Henry C. Meadow

Title Executive Secretary, Committee on Research and Development

Signature _____ Date _____

Telephone 617 - 734-3300, Extension 441
Area Code Number Extension

10035460 75

THE COUNCIL FOR TOBACCO RESEARCH – U.S.A., INC.

May 28, 1975

Grant Application No. 1030

MISCELLANEOUS

To: The committee comprising Drs. Gardner, Feldman and Sommers

Subject: Emil R. Unanue, M.D., Harvard Medical School
New application No. 1030
"Physiopathology of Normal and Activated Macrophages"

History

This applications was not handled as a Case.

Request

Application No. 1030 requests $63,490 for the first year of a three year program.

Documents submitted

1. Covering letter
2. Application dated May 8, 1975 (19 pages)
3. C.V.s of Drs. Unanue and Stadecker
4. Two reprints and one manuscript

D.S.

DS:wg
Encl.

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#1030

THE COUNCIL FOR TOBACCO RESEARCH—U.S.A., INC.
110 EAST 59TH STREET
NEW YORK, N. Y. 10022
(212) 421-8885

Application for Research Grant
(Use extra pages as needed)

Date: May 8, 1975

1. Principal Investigator (give title and degrees):

Emil R. Unanue, M.D.

2. Institution & address:

Department of Pathology
Harvard Medical School
25 Shattuck Street
Boston, Massachusetts 02115

3. Department(s) where research will be done or collaboration provided:

Department of Pathology

4. Short title of study:

Physiopathology of Normal and Activated Macrophages

5. Proposed starting date: September 1, 1975

6. Estimated time to complete: Three years

7. Brief description of specific research aims:

This application is for continuation of studies on the physiology of macrophages and their role in disease. It focuses on the investigation of biologically active molecules secreted by macrophages: their characterization, regulation, and function in physiological states and disease processes. The experimental project consists of experiments in which macrophages treated in different manners are cultured; the culture fluids are examined and characterized chemically and biologically. Work along the lines described above has been done for the past two years with positive results, some of which have been published. From this initial work, as well as from work of others, it has become quite apparent that macrophages secrete a number of powerful active molecules which have the potential of playing an important regulatory role in *in vivo* processes. Heretofore, the secretion of macrophages had been in great part ignored, yet it may represent as important a function as phagocytosis. So far we have found an inhibitor of cell proliferation and stimulatory molecules that promote lymphocyte proliferation and differentiation; others have found a number of enzymes, such as, for example, a plasminogen activator-like molecule and lysozyme.

This application contains three basic goals: attempts to 1) relate secretion of biologically active molecules with the state of activation and function of the phagocytes; 2) to isolate the molecules; and 3) to define their biological activities. We are concentrating on four activities that appear to promote a heightened immune status: differentiation of thymocytes, differentiation of B lymphocytes, increased helper activity of thymic cells, and chemoattraction.

8. Brief statement of working hypothesis:

Macrophages are cells found throughout the different tissues and endowed with powerful biological functions, mainly as concerns their role in inflammation. Macrophages respond in various ways to external stimuli and are thought capable of regulating a number of cellular functions. The role of macrophages in the lung (alveolar macrophages) in local bacterial resistance is known. Their general response to phlogogenic stimuli, such as inhaled materials, is only partially characterized. In order to properly outline the role of this cell in normal resistance, basic studies on its response are necessary. It is our contention that macrophages play an important regulatory role not only by handling of antigens but also by the elaboration and release of regulatory molecules. These molecules are best released following phagocytosis and serve to focus and increase the specific limb of the immune response, *i.e.*, the lymphocyte response. The biological and chemical characterization of these molecules secreted by macrophages may represent a fundamental and necessary step for our understanding of the function of this cell.

9. Details of experimental design and procedures (append extra pages as necessary):

Please see appended pages.

10. Space and facilities available (when elsewhere than item 2 indicates, state location):

My laboratories are in the Department of Pathology at Harvard Medical School. The Department occupies about 16,000 square feet of space. Common laboratories containing gamma and liquid scintillation counters, spectrophotomer, lyopholizer, etc., are available for all members. Ample animal facilities are found in a building next to the Department. My laboratories occupy about 2,500 square feet of space. They consist of four different, interconnecting rooms with an annex for desk space. The laboratories are fully equipped for tissue culture work, microscopy, and immunochemistry.

11. Additional facilities required:

None

12. Biographical sketches of investigator(s) and other professional personnel (append):

13. Publications: (five most recent and pertinent of investigator(s); append list, and provide reprints if available).

14. First year budget:

A. Salaries (give names or state "to be recruited")	% time	Amount
Professional (give % time of investigator(s) even if no salary requested)		
Emil R. Unanue, M.D.	20	REDACTED
Miguel Stadecker, M.D.	100	
Technical		
To be hired		REDACTED
Part-time animal caretaker		
Sub-Total for A		REDACTED
B. Consumable supplies (by major categories)		
Animals: mice, about 400 to 500 per month; rats; rabbits		$ 9,000
Chemicals and isotopes		2,400
Tissue culture supplies		6,000
Sub-Total for B		$17,400
C. Other expenses (itemize)		
Expenses for animal care and food (about $300 per month)		$ 3,600
Travel to scientific meeting		400
Sub-Total for C		$ 4,000
Running Total of A + B + C		$54,400
D. Permanent equipment (itemize)		
Hewlett Packard 65A Calcultor with programs		$ 930
Sub-Total for D		$ 930
E. Indirect costs (15% of A+B+C)		$ 8,160
Total request		$63,490

15. Estimated future requirements:

	Salaries	Consumable Suppl.	Other Expenses	Permanent Equip.	Indirect Costs	Total
Year 2		18,270	4,200	—	8,667	66,447
Year 3		19,184	4,410	—	9,206	70,582

10035460B1

16. Other sources of financial support:

List financial support from all sources, including own institution, for this and related research projects.

CURRENTLY ACTIVE

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates
Studies on Antigenic Stimulation (runs to 1978)	N.I.H. AI 10091	$64,017	01/01/75 - 12/31/75
As Co-investigator: *Experimental Cancer Immunology* (Dr. B. Benacerraf is P.I.)	N.I.H. CA 14723	38,611	06/01/75 - 05/31/76
As Co-investigator: *Ultrastructural Immunocytochemistry of Cell Surfaces* (Dr. M. J. Karnovsky is P. I.) (runs to 1978)	N.I.H. AI 10677	38,900	01/01/75 - 12/31/75

PENDING OR PLANNED

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates

It is understood that the investigator and institutional officers in applying for a grant have read and accept the Council's "Statement of Policy Containing Conditions and Terms Under Which Project Grants Are Made."

Checks payable to

Harvard Medical School

Mailing address for checks

Mr. Henry C. Meadow, 25 Shattuck Street
Boston, Massachusetts 02115

Principal investigator

Typed Name Emil R. Unanue, M.D.

Signature E. R. Unanue Date May 8, 1975

Telephone 617 (Area Code) 734-3300 (Number) 364 (Extension)

Responsible officer of institution

Typed Name Mr. Henry C. Meadow

Title Executive Secretary, Committee on Research and Development

Signature Henry C. Meadow Date 5.13.75

Telephone 617 (Area Code) 734-3300 (Number) 441 (Extension)

10035460 82

Background Information

The literature on the functions of phagocytes (*i.e.*, monocyte-macrophages) in physiological and pathological states is extensive. It is not my intention to review it but to emphasize the major points relevant to this application having to do with the macrophage and its role in resistance and immunity. Macrophages participate to some degree in a wide number of processes, all of which deal with the inflammatory reaction against undesired materials. These cells are endowed with a number of properties that allow them to have a central role in inflammation (1): 1) macrophages distribute widely throughout various tissues; all the evidence indicates that they originate from a rapidly proliferating precursor found abundantly in bone marrow; from this precursor phagocytes differentiate and home to sites of inflammation—the differentiation and homing is under some control which has as yet to be defined in precise terms. 2) Phagocytes have membrane properties that enable them to bind a large number of materials; in particular, phagocytes take up a wide number of antigen molecules via "nonspecific" sites (*i.e.*, not characterized surface components) and by surface receptors for the Fc portion of Ig and for activated C3; following uptake, phagocytes interiorize and effectively degrade most of the foreign material (3). Hence, phagocytes play an essential role in elimination of antigens. Lastly, 3) phagocytes respond to environmental stimuli by becoming "activated"; activated phagocytes have increased biosynthesis of enzymes, are more active in phagocytosis, and are more microbicidal (4). (The term "activated" is a very poor one, meaning different things to different investigators. I used it to denote the macrophages that increase their metabolic function in response to external stimulation.) As a result of these three main properties, phagocytes

represent a pivotal cell in the inflammatory process and in immunity. *In inflammation*—in general—phagocytes are believed to play a major role in elimination of dead material and in wound healing. *In immune induction*, the role of phagocytes is of considerable importance. These cells, by handling and focusing antigen molecules play a crucial regulatory role in induction in part determining the size of the immunogenic stimulus (5). The uptake of antigen by macrophages represents a crucial step in the initiation of a full immune response. *In the efferent arc of immunity*, phagocytes represent the main cellular component of cellular type of immunities. Macrophages undoubtedly represent the major cellular component involved in resistance to certain infectious diseases, such as those produced by facultative intracellular bacterias (4). Their role in the processes is essential. A great unanswered question concerns the role of macrophages in general resistance to tumors. Two sets of observations point to some kind of a role, although this is still to be determined: 1) administration of certain live bacteria at the site of a tumor produces a marked infiltration with macrophages and a reduction of tumor growth—evidence would indicate that this process cannot be explained only by specific anti-tumor immunity (6); 2) in *in vitro* situations, macrophages have been found to exercise a cytostatic or cytocidal effect on tumors. These two series of observations suggest some kind of control of macrophages on cellular growth (7).

The exact manner by which macrophages exert their different functions is not clear. First, it is obvious that one set of effects is directly related to their capacity for endocytosis and intracellular elimination of foreign materials. A wealth of information is available on this process, which I will not detail since it is not the intention of this proposal to

study it. Very recently, however, it has become apparent that macrophages exert some biological control by secreting certain molecules into the extracellular environment. This function of macrophages was first indicated by *in vitro* experiments in which some lysosomal enzymes were found to be released into the outside medium following phagocytosis, suggesting to some that this release could be of significance in producing tissue damage (8). The process of secretion became, however, very evident for certain enzymes or bactericidal molecules, such as a plasminogen activator-like molecule or lysozyme (9, 10). These two molecules, in contrast to lysosomal enzymes, were destined mainly for export, *secretion*, and were not retained by the macrophage for intracellular handling, as it happened with lysosomal enzymes.

Our laboratory has been interested in the pathophysiology of macrophages mainly as it concerned the role of these cells in immune reactions. It is very obvious in a number of experimental systems—*in vivo* and *in vitro*—that macrophages exert an important regulatory role in immune induction. This role has been explained, in part, by the capacity of these cells to concentrate antigen and present a small finite number of undegraded molecules to the lymphocyte. In other words, macrophages apparently function as an antigen-focusing cell favoring the interaction of various collaborating lymphocytes with antigen. This helper function contrasts markedly with the phenomena observed *in vitro* in which macrophages stop the growth of tumor cells. Furthermore, it has been reported that macrophages may also play a detrimental effect on growth of non-neoplastic cells, including lymphocytes.

During the past year we set up a series of experiments to reappraise the effects of macrophages on various cells in culture. Our first experimental

design, which proved to be successful, was simply to culture macrophages under various conditions, *i.e.*, normal or activated, for various periods of time, following or not phagocytosis, etc., and then to test the culture fluids for their effects on other cells. It became immediately obvious that a number of active molecules were being secreted, some of which had contrasting effects on cellular growth. More importantly it appeared in preliminary experiments that the secretion of these molecules was regulated by the activity of the macrophages. The *in vitro* effects of the secreted material were quite dramatic. Indeed, their biological potential cannot be underestimated.

The first molecule to be found was a low-molecular-weight compound (about 600 daltons) that inhibited protein and DNA synthesis of various kinds of cells (11). The inhibitor was best seen in high-density cultures of macrophages. A number of cells, including neoplastic ones, cultured in medium containing the inhibitor did not synthesize DNA but were viable for at least 24 hours. The inhibitor was synthesized by the macrophages in culture, became bound to target cells, inhibited not only tumor cells but also lymphocyte proliferation and differentiation. This inhibitor was secreted only by macrophages; it was not found in cultures of lymphocytes, fibroblasts, or other cells. We have as yet no chemical definition of it. Preparative work has been in progress with Professor Manfred Karnovsky in the Department of Biochemistry at this Medical School. The important question of the relationship between its secretion and the activity of the macrophage has not been resolved yet. It is clear, however, that the material is found in cultures of both normal macrophages and macrophages activated by *Listeria* infection. My thoughts are that this molecule could conceivably play a biological role only in conditions where a large number

of macrophages focus around target cells, such as it occurs in immune granulomas. If not, it is difficult to visualize a role because of its very small size. Nevertheless, the biochemistry and mode of action are a fascinating biological problem. The presence of this inhibitor explains some of the cytostatic effects of macrophages on tumor cells seen in culture. It may also explain some of the results seen *in vivo* when certain adjuvants like BCG are injected with tumors, producing a marked macrophage infiltration and a reduction of tumor growth. Studies on this molecule are in progress.

The second set of molecules were found following removal by dialysis of the low-molecular-weight inhibitor (12, 13). Indeed, following dialysis (or in undialyzed medium from low-density cultures), we found molecule(s) that stimulated lymphocyte growth and differentiation. The biological activity of these sets of molecules were shown on several assay systems, all *in vitro*: 1) thymocytes were stimulated to proliferate and to respond to mitogens; 2) B lymphocytes, the precursor of antibody-forming cells, were stimulated to differentiate into plasma cells; 3) T lymphocytes increased their helper activity. For example, a dramatic effect of these macrophage factors (abbreviated MCF) was seen in cultures of spleen cells from athymic mice which could be made to respond immunologically to antigen. We have submitted a paper, now in press in the Journal of Experimental Medicine, which I enclose and which summarizes these points. This paper contains the basic methodology and the results on which this proposal is grounded. Of great interest are the following observations: 1) in the preliminary experiments, it was found that phagocytosis of particles *stimulated secretion* of twenty to one-hundred times more material; 2) in the only experiment done, chemical fractionation of the material suggests that there is more

than one molecule involved: the thymocyte stimulatory molecule is about 16,000 daltons, while the activity that stimulates B and T cells falls both into the 15,000 range as well as into the range of 100,000.

At the same time these studies were conducted, we decided to search also in macrophage culture fluids for activities that would attract cells to macrophages. It was our thought that if macrophages play a role in inductive events by antigen presentation as well as in resistance to infection, then certain mechanisms should be operative in bringing lymphoid, as well as other cells to foci of macrophages where antigen was being concentrated. Suggestive evidence to a relationship between antigen trapping by macrophages and lymphoid cell accumulation came from observations that the earliest cellular reaction to the entrance of antigen into a lymph node is an accumulation of lymphocytes. Indeed, radiolabeled lymphocytes accumulate into a node when particulate materials enter and are trapped by macrophages (14). Our early studies tested whether macrophage culture fluids obtained before or after phagocytosis would contain a molecule that attracted lymphocytes. We did the experiment in the rat, the assay for chemotaxis being done by Dr. Peter Ward of the University of Connecticut. We found that macrophage culture fluid *after phagocytosis* did contain a powerful chemotactic agent for lymphocytes. The importance of this observation, if true, is obvious since it places the macrophage at the time of phagocytosis in a central position regulating cell traffic.

Experimental Protocol

The purpose of this request is to further extend our analysis of biologically active molecules secreted by macrophages. At the present time we do not know how many different molecules are involved and are

being secreted, nor their biochemistry and precise mode of action. We have no idea of their role in *in vivo* processes nor do we know the mechanism controlling their biosynthesis and secretion. The long-range goals are to identify the secreted molecules in biochemical and biological terms. I now outline projects that represent a continuation of the present experiments: investigate: 1) the relationship between activity of macrophages and secretion of active products; 2) the biochemistry of the secreted molecules; and 3) their biological characterization.

Most technical details are included in the enclosed reprint (No. 3), which details our rationale for using the different assay systems of antibody formation. The basic technique is to obtain culture fluids from macrophages. These culture fluids at various dilutions are tested for their effects: 1) in stimulating DNA synthesis of thymocytes; 2) in increasing the helper activity of T lymphocytes in a hapten-carrier system *in vitro*; this is done by culturing spleen cells from selected mice immune to a hapten protein, in our case fluorescein (F) conjugated to hemocyanin (KLH), with the same antigen; after four days, the number of antibody-forming cells made to F is determined by a Jerne plaque assay (the spleen of the immune mice will contain antibody-forming cell precursors—B cells—reactive to F and ready to respond if challenged with the antigen, provided that T lymphocytes with the carrier protein, KLH, come into the system as helper cells—this being the classical setup of B-T cell interaction but now being modulated by products of phagocytes). 3) In producing differentiation of B cells; antibody-forming cell precursors cultured in MCF in the absence of T helper cells differentiate to plasma cells; we tested this by cultured primed spleen cells to F-KLH in the presence of F in an unrelated carrier protein (rabbit gamma globulin); and 4) chemotaxis using modified Boyden chambers as per conventional methods. All these studies will be done on macrophage fluids following dialysis to remove the inhibitor. I do plan to include analysis of it in undialyzed fluids.

A) The first project questions whether macrophages stimulated in various ways synthesize and secrete different amounts (or classes) of molecules. I believe it is important to outline the conditions, cellular or humoral, that may modulate the phenomenon of secretion. By doing this we will be in a strong position to place the phenomenon in a better

perspective and perhaps obtain an idea of its true biological significance. It is known that some responses of the macrophages are under modulation by environmental influences—indeed, phagocytosis, for example, induces more synthesis of lysosomal enzymes; infections associated with cellular immunity, likewise, lead to macrophage activation in terms of increased bactericidal activity. [illegible] investigate 1) the relationship between activity [illegible]

The idea is to carry out the experiments in the mouse (and/or rat). Macrophages will be isolated and stimulated *in vitro* by exposing them to a series of materials—most of the materials selected will be those that are readily taken up by the cell; included are those that result in marked adjuvant type of effects. Included are simple materials that are readily taken up by phagocytosis, such as latex particles, antigen-antibody complexes made up of soluble protein, or particulate antigens (such as sheep erythrocytes), and various dead bacteria. Of importance is to consider certain bacteria known to produce marked stimulation of macrophages (and also of immune responses): tubercule bacillus, *Corynebacterium parvum* and *Listeria monocytogenes*; and nonbacterial adjuvants, such as beryllium salt. Depending on the results, we will try to obtain an idea if the reaction is modulated by the step of membrane-particle interaction prior to phagocytosis or by the phagocytic process itself or by the *nature* of the material. This can be done by varying size and nature of the phylogogenic material (for example, antigen-antibody complexes can be attached to the surface of the culture dish; the macrophages will not ingest them, but still their surface receptors will interact with them. In these conditions, is secretion stimulated?

In summary, macrophages are to be exposed to the various materials and cultured for various time periods; culture fluids are then removed and tested biologically. Morphological and cytochemical determinations (for [illegible] acid phosphatase and total cell protein) will also be determined.

Another variant of this experiment is to administer the phlogogenic materials *in vivo* and then to determine if the spleen or peritoneal macrophages secrete more of the biologically active products.

It is important to consider that there may be more than one stimuli needed to produce an effect. Perhaps macrophages need to be activated

first (as it results, for example, by a systemic bacterial infection in which bacterial products plus the immune reaction produces system activation of all phagocytes) and then challenged by a phagocytic event. These kinds of relationships are amenable to exploration using macrophages exposed *in vivo* and *in vitro* or both in combination. The use of macrophages from different organs, including lung and spleen, is to be considered.

An important point to analyze along these lines is the relationship between secretion and biosynthesis of the active products. This will be tested in cultured macrophages in which protein synthesis is stopped following treatment with the various protein synthesis inhibitors.

In essence, we first plan a series of experiments testing whether various stimuli signal the macrophage to make and/or secrete the modulatory molecules. The experiments should give us an indication whether this function is controlled by external stimuli and of some of its basic mechanisms.

B) A second important goal is the chemical isolation and characterization of the molecules. This will be attempted by preparative and analytical methods such as electrophoresis or column chromatography. (Figure 5 of the enclosed paper No. 3 shows our initial attempt.) We have sufficient experience to be able to carry out at least part of the initial biochemical work. Our first idea is to culture macrophages in medium devoid of fetal calf serum (see the experiment of Figure 5 for an analysis of this point), concentrate the fluids, and attempt separation of the molecules by Sephadex G200 or G100 filtration. Chemical analysis of the purified or enriched materials will be done using polyacrylamide gel electrophoresis; the experiments also call for the sensitivity of the material to various enzymes, including proteolytic ones. My laboratory

does collaborative work with that of Prof. Manfred Karnovsky in the Department of Biochemistry here. Dr. Karnovsky has a large interest in these matters involving biochemistry of phagocytic functions and in continuously advising us in these matters. The ultimate goal of precise analysis will obviously require his assistance or that of members of his department. Organs, including lung and spleen, is to be considered.

C) The third project concerns itself with the biological characterization of secreted molecules. Several lines of investigation are contemplated. The first series of experiments described in the attached manuscripts suggests that one molecule has the capacity to increase or develop thymic function—thymocytes proliferate and are able to respond to phytohemagglutinin (immature thymocytes respond poorly, if at all, to PHA). This suggestion is strengthened by the results shown with the spleen cells of athymic mice that strikingly respond immunologically when cultured with antigen in the macrophage fluids. It is possible that the undifferentiated stem cells of the nude athymic mouse are rapidly stimulated to differentiate into T helper cells. We plan to test whether the MCF promotes differentiation of thymocytes (and stem cells formed in spleens of athymic mice). This will be done by: a) culturing the cells in MCF and assaying cytochemically for content of several thymic alloantigens—the thymocyte, as it differentiates changes its surface macromolecules, the alloantigen θ decreases, H-2 antigen increases; b) assaying culture cells for these immunological functions of mature T cells, *i.e.*, graft-*versus*-host type of assays and capacity to help B cells for antibody formation. The possibility that the MCF, likewise, promotes B cell differentiation to plasma cells was suggested by results in which enriched B cell populations cultured in MCF differentiate to secreting cells. This experiment will be further explored—B cells from

macrophages immunized to two or three antigens (horse red cells, the hapten F, or DNP) will be isolated by preparative methods, cultured at various times in the presence of MCF, and assayed for antibody-forming cells at various time intervals. By doing this we should obtain an idea whether the MCF in the presence or absence of antigen and in the absence of thymic helper cells promotes differentiation to plasma cells and the time required to do it.

The studies of chemotactic material consists of tests for chemotaxis using modified Boyden chambers and following conventional methods. As target cells, we will employ macrophages, lymphocytes (as whole populations or as semipurified B or T), and neutrophils. We, therefore, hope to establish whether there is a single chemotactic material and its cellular specificity.

All the experiments so far detailed call for analysis of these powerful secreted molecules and their relationship to macrophage function. The crux of the matter, however, is whether these molecules are operative in *in vivo* or simply represent a laboratory curiosity. The point holds true actually for many kinds of mediators of inflammation and of immunological reactions described so far, and its solution is not an easy one to tackle.

I plan to approach this problem along two lines of investigation: 1) to try and develop an antibody to the molecules in question and by doing this use the antibody as a probe to determine whether the molecules are found *in vivo* or whether the antibodies will modify particular macrophage functions. Making antibodies to soluble mediators has been very difficult in the past because most of the mediators are poor immunogens, apart from the fact that biochemical purity has not been accomplished. One hopeful point in favor of success in our experiments is that the macrophage

stimulatory molecules are found in culture fluids basically free of extraneous materials such as fetal calf serum. Our idea is to obtain the "purified" fractions from the experiments detailed in Project 2 and immunize rabbits repeatedly. I plan to follow conventional approaches, trying to use as pure a material as possible and doing the series of immunization gimmicks familiar to immunologists (*i.e.*, incorporation of the antigen into adjuvants like Freunds and/or attaching the antigen to a schlepper carrier). The antiserum will then be tested for its capacity to neutralize the activity *in vitro* before proceeding to any *in vivo* analysis of its effects.

2) The second approach is to test the MCF for the biological activity directly *in vivo*. That is to say, if these factors are also operative *in vivo*, one would expect that simple introduction of antigens with them should modify a regular immune response. Experiments testing conventional immune responses to antigen given with or without the MCF are thus contemplated. In particular, we are keen in testing athymic mice. We know from the *in vitro* data that spleen cells from these mice are made to respond to the MCF. Hence, the nude athymic mice may become the best host for examining this problem.

In summary, based on our initial experiments we know that macrophages secrete into the extracellular milieu a number of molecules having powerful modulating activity on various cells including lymphocytes. Our hypothesis is that the initial step in immunity involves the uptake of antigen by the phagocytes and from there the whole series of inductive events rapidly ensues, comprising a series of amplification steps and control mechanisms. Involved among the amplification steps may be the series of molecules described herein, all having as a function to trigger best the lymphocyte

which is the specific arm of the response. This proposal, then, represents an effort to understand further macrophage function and its role in local and generalized resistance and immunity. We should not overlook the potential use of these molecules in future clinical trials.

References

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3. Cohn, Z. A. Adv. Immunol., *9*:97, 1968.
4. Mackaness, G. B. J. Exp. Med., *129*:973, 1969.
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14. Zatz, M. M., and Lance, E. M. J. Exp. Med., *134*:224, 1971.

Curriculum Vitae—Emil Raphael Unanue

Born: REDACTED

	B.S., Institute of Secondary Education
	M.D., University of Havana School of Medicine
1961 to 1962	Intern in Pathology, Presbyterian University Hospital, Pittsburgh, Pennsylvania
1962 to 1965	Research Fellow, Department of Experimental Pathology, Scripps Clinic and Research Foundation, La Jolla, California
1966 to 1968	Research Fellow, Immunology Division, National Institute for Medical Research, London, England
1968 to 1970	Associate, Department of Experimental Pathology, Scripps Clinic and Research Foundation, La Jolla, California
1970 to 1971	Assistant Professor of Pathology, Harvard Medical School, Boston, Massachusetts
1972 to 1974	Associate Professor of Pathology, Harvard Medical School, Boston, Massachusetts
1974	Mallinckrodt Professor of Immunopathology, Harvard Medical School, Boston, Massachusetts

Memberships

1966	REDACTED
1966	
1967	
1974	REDACTED

Honors and Award

1962	Recipient, Certificate of Education Council for Foreign Medical Graduates, U. S. A.
1966 to 1969	Fellow of Helen Hay Whitney Foundation
1968	Recipient, T. Duckett Jones Award of Helen Hay Whitney Foundation
1969 to 1970	Senior Fellow of the American Cancer Society, California Division
1971	Recipient, Research Career Developmental Award, National Institutes of Health
1973	Parke Davis Award, American Society for Experimental Pathology

Other Academic Activities

1972 Associate Editor, Journal of Immunology
1972 Associate Editor, Clinical Immunology and Immunopathology
1973 Associate Editor, International Archives of Allergy and Applied Immunology
1973 Member, Pathology A Study Section, National Institutes of Health
1974 Associate Editor, Journal of the Reticuloendothelial Society

Total publications: 96

Representative papers:

1. Unanue, E. R., and Cerottini, J.-C. The immunogenicity of antigen bound to the plasma membrane of macrophages. J. Exp. Med., *131*: 711, 1970.

2. Cruchaud, A., and Unanue, E. R. Fate and immunogenicity of antigens endocytosed by macrophages: a study using foreign red cells and immunoglobulin G. J. Immunol., *107*:1329, 1971.

3. Unanue, E. R. The regulatory role of macrophages in antigenic stimulation. Adv. Immunol., *15*:95, 1972 (summarizes all our work with macrophages to 1972).

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5. Katz, D. H., and Unanue, E. R. Critical role of determinant presentation in the induction of specific responses in immunocompetent lymphocytes. J. Exp. Med., *137*:967, 1973.

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7. Unanue, E. R., and Katz, D. H. Immunogenicity of macrophage-bound antigens: The requirement for hapten and carrier determinants to be on the same molecule for T and B lymphocyte collaboration. Eur. J. Immunol., *3*:559, 1973.

Representative papers, continued

8. Unanue, E. R. Cellular events following binding of antigen to lymphocytes. (Parke-Davis Award Lecture) Am. J. Pathol., *77*:2, 1974.

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11. Calderon, J., Kiely, J.-M., Lefko, J. L., and Unanue, E. R. The modulation of lymphocyte functions by molecules secreted by macrophages. I. Description and partial biochemical analysis. J. Exp. Med., in press, 1975 (enclosed as Reprint No. 3).

Curriculum Vitae

Miguel J. Stadecker

Born: REDACTED

Family: REDACTED

Education:

R B.S., National School Nr. 8, Buenos Aires, Argentina

R M.D., University of Buenos Aires School of Medicine, Buenos Aires, Argentina

1967-1970: Instructor in Pathology, University of Buenos Aires School of Medicine, Buenos Aires, Argentina

Assistant Pathologist, Ramos Mejia Hospital, Buenos Aires, Argentina

1970-1971: Second Assistant Resident, Department of Pathology, Massachusetts General Hospital, Boston, Massachusetts

1971-1972: Third Assistant Resident, Department of Pathology, New England Medical Center Hospital, Boston, Massachusetts

Instructor in Pathology, Tufts University School of Medicine, Boston, Massachusetts

1972-1973: Chief Resident, Pathology, New England Medical Center Hospital, Boston, Massachusetts

Instructor in Pathology, Tufts University School of Medicine, Boston, Massachusetts

Graduate Student in Immunology, Tufts University School of Medicine, Boston, Massachusetts

1973-1974: Fellow in Immunology-Pathology, Tufts University School of Medicine, Boston, Massachusetts

Instructor in Pathology, Tufts University School of Medicine, Boston, Massachusetts

Candidate for the degree of Ph.D. in Immunology, 1975, Tufts University.

Languages: English, Spanish, German, French

10035461 00

Honor Societies: Argentine Medical Association
Argentine Society of Pathologists
International Academy of Pathology

Examinations: E. C. F. M. G., September, 1969
American Board of Pathology, Anatomic-Diplomate, November, 1973

Publications

1. Stadecker, M. J. The normal lymph node, a review. Rev. Fis. Ter. Clin. (Buenos Aires), 2:5, 1970.
2. Stadecker, M. J., and Leskowitz, S. The cutaneous basophil response to particulate antigens. P.S.E.B.M., 142:150, 1973.
3. Stadecker, M. J., Bishop, G., and Wortis, H. H. Rosette formation by guinea pig thymocytes and thymus-derived lymphocytes with rabbit red blood cells. J. Immunol., 111:1834, 1973.
4. Braylan, R., Pascuccelli, H., Stadecker, M. J., and Morgenfeld, M. Hodgkin's disease—A report from Buenos Aires, Argentina. Cancer, 32:879, 1973.
5. Stadecker, M. J., and Leskowitz, S. The cutaneous basophil response to mitogens. Fed. Proc., 33:733, 1974.
6. Stadecker, M. J., and Leskowitz, S. The cutaneous basophil response to mitogens. J. Immunol., 113: Aug. 1974.

1003546102

#1042-YOSHINAGA

THE COUNCIL FOR TOBACCO RESEARCH–U.S.A., INC.

July 7, 1975

Grant application No. 1042

MISCELLANEOUS

To: The committee comprising Drs. Bing, Gardner and Meier

Subject: Kohi Yoshinaga, Ph.D., Harvard Medical School, Boston
New application No. 1042
"Effects of Nicotine on Pregnancy"

History

An informal inquiry was handled as Case 311 and encouraged.

Request

Application No. 1042 requests $33,120 for the first year of a three year project. Estimates for the second and third years are $35,075 and $36,915, respectively.

Documents submitted (attached)

1. Application dated June 27, 1975 (14 pages including C.V. of Dr. Yoshinaga).

2. Five reprints.

D.S.

DS:wg
Att.

+1023

THE COUNCIL FOR TOBACCO RESEARCH–U.S.A., INC.
110 EAST 59TH STREET
NEW YORK, N. Y. 10022
(212) 421-8885

RECEIVED
JUL 2 - 1975

Application for Research Grant
(Use extra pages as needed)

Date: June 27, 1975

1. Principal Investigator (give title and degrees):
Koji Yoshinaga, Ph.D.
Associate Professor of Anatomy

2. Institution & address:
Laboratory of Human Reproduction and Reproductive Biology
Harvard Medical School
45 Shattuck Street, Boston, Massachusetts 02115

3. Department(s) where research will be done or collaboration provided:
Laboratory of Human Reproduction and Reproductive Biology

4. Short title of study:
Effects of nicotine on pregnancy.

5. Proposed starting date: January 1, 1976
6. Estimated time to complete: 3 years
7. Brief description of specific research aims: Nicotine has been reported to exert deleterious effects on pregnancy. Nicotine acts not only on the genital tract to alter its movement, but also on the pituitary gland to inhibit the secretion of luteinizing hormone and prolactin. Since these two hormones play important roles in stimulating ovarian hormone secretion, the deleterious effects of nicotine on pregnancy may be through the hypothalamo-pituitary-ovarian axis. The aims of the proposed research are to determine if nicotine exerts direct action on the genital tract-embryo and/or indirect action on the endocrine system and to clarify the mode of action of nicotine on pregnancy with particular emphasis on hormone imbalance in the hypothalamo-pituitary-ovarian axis caused by nicotine.

8. Brief statement of working hypothesis:

Inhibitory action of nicotine on the secretion of luteinizing hormone may be mediated by inhibition of release and/or production of gonadotrophin releasing hormone in the hypothalamus. Suppression of the secretion of luteinizing hormone and prolactin by nicotine will result in subnormal secretion of progesterone and estrogen by the ovary and normal progress of pregnancy will be interfered. If nicotine acts mainly on the endocrine system, supplement of nicotine treated-animals with ovarian hormones will overcome the deleterious effects of nicotine on pregnancy. If they are not overcome, direct effects of nicotine on the genital tract and/or embryo will become obvious.

9. Details of experimental design and procedures (append extra pages as necessary)

Introduction

Although cigarette smoking has been reported to exert deleterious effects on pregnancy (1,2), few analytical studies have been done on the mode of action of inhaled substances. It has not been determined whether the deleterious effects of nicotine on pregnancy (3) are direct on the genital tract-embryo or indirect on the endocrine system which regulates the reproductive processes (the hypothalamo-pituitary-ovarian axis).

In early pregnancy movement of cilia and muscle of the genital tract facilitates transport of fertilized ova through the Fallopian tube and location of ova in implantation sites of the uterus. It has been shown that the cilial movement of the Fallopian tube and muscle contraction of of the Fallopian tube and the uterus are influenced by ovarian hormones, estrogen and progesterone (4,5). Although nicotine alters the contractile activity of the genital tract, the effect of nicotine is still influenced by estrogen and progesterone (6). If a small amount of estrogen is administered to pregnant mice while fertilized ova are in the Fallopian tube, transport of the ova is blocked and the ova are retained in the Fallopian tube for a prolonged period of time (7). Since nicotine affects contracti activity of the Fallopian tube (6), nicotine may alter the speed of ovum

transport resulting in its untimely arrival into the uterus. We have much evidence that asynchrony of the ovum arrival and preparation of the uterus to receive the ovum often result in unsuccessful implantation of the ovum (8,9,10). Prolonged gestation period in nicotine treated rats observed by Becker et al. (3) may be due to a delay in ovum implantation: this phenomenon is frequently seen in the rat whose estrogen secretion is hindered by agents such as tranquilizers(11) or reserpine (12) and under the condition of concurrent lactation(8) and stress (13).

It has been shown that nicotine delays and suppresses the secretion of luteinizing hormone (14) and prolactin (15). Since these two hormones stimulate, with various combinations, progesterone secretion (18,19,20), suppression of luteinizing hormone and prolactin will reduce progesterone secretion (19, 21). Luteinizing hormone has also been shown to stimulate estrogen secretion (22). When luteinizing hormone level is lowered by neutralizing with its antibody, estrogen secretion is supppressed (23).

A study on placental transfer and distribution of nicotine in the fetus shows that nicotine concentration in fetal circulation is higher and its clearance rate slower than those in the mother (24). Thus direct effect of nicotine on the embryo cannot be eliminated.

In this proposed research we aim to analyze the effects of nicotine on pregnancy by determining which hormones in the hypothalamo-pituitary-ovarian axis are suppressed by nicotine. The obtained results will be correlated with deleterious effects of nicotine on other biological parameters. During the first year we will concentrate our effort on the period between ovulation and ovum implantation. Studies on later stages of pregnancy will be carried out during the subsequent years.

Experimental design

The rat will be used in this study as an experimental animal. Pregnant rats treated with various doses of nicotine will be sacrificed (6 rats in a group) at 3 hour intervals from day 0 to day 6 of pregnancy to collect samples for measurement of hormones and other biological parameters. The hormones to be measured are: gonadotrophin releasing hormone (also called as luteinizing hormone releasing hormone)in the hypothalamus; luteinizing hormone, follicle stimulating hormone and prolactin in the pituitary gland and serum; and ovarian steroid hormones in the serum (progesterone, 20α-hydroxypregn-4-en-3-one,estradiol and estrone).

Other biological parameters are: location, appearance and viability of ova; number of implantation sites in the uterus; number and weight of corpora lutea; and time of ovum implantation.By locating the ova in the genital tract at various stages of pregnancy, we can estimate the speed of ovum transport. Appearance of ova (the size and number of blastomeres) will reveal their developmental stages or degree of degeneration. The number of corpora lutea will be considered as the number of ova ovulated. This number will be used for calculation of the percentage of ova developed to various stages of embryonic development. Viability of ova will be examined by determining their ability to develop after transfer into the uterus of recipients (pseudopregnant rats).

The mode of action of nicotine will be deduced from comparison of the secretory pattern of hormones with other biological parameters. The obtained conclusions will be tested by determining if compensation of the reduced hormone will overcome the nicotine effect. If the hormone therapy does not overcome the nicotine effect, nicotine is considered to have exerted direct effect on the genital tract and/or embryo.

Procedure

1. Animals

Young adult female rats (60 days old) will be purchased from Charles River Breeding Laboratories, Wilmington, Mass.. The estrous cycles will be traced by vaginal smear method and the rats at pro-estrous stage will be placed with fertile males overnight. The vaginal smear will be examined in the following morning; those rats with spermatozoa are considered pregnant and this day will be designated as day 1 of pregnancy.

2. Treatment of rats with nicotine

From day 0 (the day of proestrus) the rats will be injected subcutaneously twice daily (at 900 hr and 1800 hr) with high, intermediate or low dose of nicotine (5, 1 or 0.2 mg/ day; namely 15, 3 or 0.6 mg nicotine tartrate) disolved in saline. Control rats will receive the vehicle only (0.9% NaCl).

3. Collection of samples

The rats will be sacrificed by decapitation at 3 hour intervals from 900 hr on day 0 till 900 hr on day 6 of pregnancy. Implantation of the ovum normally takes place in the afternoon of day 5.

Immediately after decapitation blood will be collected from neck blood vessels. Serum will be separated by centrifugation after clot formation. The hypothalamus and pituitary gland will be collected from the head and will be frozen on dry ice as soon as possible. The ovaries, Fallopian tubes and uterus will be dissected out. The Fallopian tubes and the uterus will be flushed with saline for collection of ova according to the method previously reported (25) and to that of Dickmann (26). The number of ova and their size and appearance

(developmental stage or degree of degeneration) will be recorded. The numbers of corpora lutea and implantation sites are also recorded. The sites of ovum implantation at very early stages (afternoon of day 5) will be visualized by a blue dye injection (27).

4. Measurement of hormones

A. Gonadotrophin releasing hormone (GnRH).

A radioimmunoassay method for GnRH reported in our earlier publications (28,29) will be used. The hypothalamic area which was cut out immediately after decapitation and kept frozen will be homogenized in 1 ml 0.2 M ice chilled acetic acid. The homogenate will be stored for 24 hr at 4 C and cenrifuged at 20,000 Xg for 1 hr. Duplicate aliquots of 200 µl supernatant will be neutralized with 200 µl 0.2M NH_4OH. After addition of 400 µl 0.2M tris-acetate buffer (pH 7.3), 100 µl antiserum to GnRH (1:300-1:1,000), and 100 µl ^{125}I-GnRH, the mixture will be incubated for 4 hr at room temperature and subsequently 44 hr at 4C. After incubation antigen-antibody complex will be separated by precipitation of unreacted labeled GnRH with dextran T70-coated charcoal. The supernatant will be subjected for counting.

B.Pituitary hormones: luteinizing hormone(LH), follicle stimulating hormone (FSH) and prolactin.

LH, FSH and prolactin will be measured by radioimmunoassay using NIAMDD (National Institute of Arthritis and Metabolic and Digestive Diseases) kits. The pituitary will be homogenated in phosphate buffered saline. The tissue concentration in the homogenate will be 10 mg wet weight/ml. The extract will be assayed for LH, FSH and prolactin. These hormones in the serum will be likewise assayed.

C. Ovarian steroid hormones:

Steroids in serum will be extracted with ether and progesterone, 20α-hydroxypregn-4-en-3-one(20α-OH-P), estradiol and estrone will be separated by Sephadex LH-20 column chromatography. 20α-OH-P will be converted to progesterone by chromic acid oxidation. In our study the values obtained for 20α-OH-P by radioimmunoassay were within the comparable range of those measured by gas liquid chromatography(19).

5. Viability test of ova

In order to examine if nicotine acts directly on the ovum and affects its later development, ova will be collected from the Fallopian tube or the uterus of nicotine treated rats and transferred to untreated recepients according to the method described in an earlier publication (25). Pseudopregnant rats will be used as the recipients of the ova. Pseudopregnancy will be induced by mechanical stimulation of the uterine cervix on the day of estrus(the last day of vaginal cornification is designated as day 1 of pseudopregnancy). The day of pseudopregnancy and the age of the ova will be synchronous.After transfer of the ova the recipients will be laparotomized on days 9, 14 and 19 of(pseudo-)pregnancy and number of implantation sites,and developing fetuses will be recorded.

6. Hormone therapy of the nicotine treated rats

From the data obtained from hormone measurement and other biological parameters we will know which hormone is suppressed by nicotine at what stage of pregnancy. Since suppression of hormones at higher levels (hypothalamus and pituitary) will be reflected by suppression of ovarian steroids, nicotine treatment is expected to result in a reduction of estrogen or progesterone. In order to find out if

supplement of reduced hormone(s) will overcome the nicotine effect, estrogen and/or progesterone will be administered to nicotine treated rats and subsequent embryonic development will be studied. The dose of hormones and period of treatment will be decided after obtaining the data on hormone levels.

References

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3. Becker, R.F., J.E. King and C.R.D. Little (1968) Am. J. Obstet. Gynec. 101, 1109.
4. Boling, J.L. (1969) In Mammalian Oviduct. Eds. E.S.E. Hafez and R.J. Blandau, The Univ. Chicago Press, Chicago, p.163.
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8. Yoshinaga, K. (1961) J. Reprod. Fert. 2, 35.
9. Yoshinaga, K. and R.O. Greep (1971) Endocrinology 88, 627.
10. Yoshinaga, K. and R.O. Greep (1974) In Progress in Reproduction Research and Pouplation Control. Eds. S.M. Husain and A.F. Guttmacher, Publication Internat., Quebec, p.137.
11. Mayer, G.(1964) In Techniques in endocrine research. Eds. P. Ecksteii and F. Knowles, Acad. Press, New York. p.245.
12. Barraclough, C.A. and C.H Sawyer (1959) Endocrinology 65, 341.
13. ~~Chambon, Y.~~ Canivenc, R. and G. Mayer (1955) C. r. Acad. Sci. 240, 1273
14. Blake, C.A. R.J. Scaramuzzi, R.L. Norman, S. Kanematsu and C.H. Sawyer (1972) Proc. Soc. Exper. Biol. Med. 141, 1014.
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18. Yoshinaga,K.and J.J. Ford (1974) In Gonadotropins and gonadal function, Ed. N.R. Moudgal, Aaad. Press New York, p.260.
19. Ford, J.J. and K. Yoshinaga (1975) Endocrinology 96, 329.
20. Ford, J.J. and K. Yoshinaga (1975b) Endocrinology 96, 355.
21. Yoshinaga, K., N.R. Moudgal and R.O. Greep (1971) Endocrinology 88, 1126.
22. Macdonald, G.J., D.T. Armstrong and R.O. Greep (1967) Endocrinology 80,.172.
23. Madhwa Raj, H.G., M.R. Sairam and N.R. Moudgal (1968) J. Reprod. Fert. 17, 335.
24. Suzuki, K., T. Horiguchi, A.C. Comas-Urrutia, E. Mueller-Heubach, H.O. Morishita and K. Adamsons (1974) Am J. Obstet. Gynec. 119,253.

25. Yoshinaga, K. and C.E. Adams (1966) J. Reprod. Fert. 12, 583.
26. Dickmann, Z. (1971) In Methods in Mammalian Embryology. Ed. J.C. Daniel, Jr. W.H. Freeman & Co. San Francisco, p. 133.
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28. Makino, T., M. Takahashi, K. Yoshinaga and R.O. Greep (1973) Contraception 8, 133.
29. Takahashi, M., J.J. Ford, K. Yoshinaga and R.O. Greep (1975) Endocrinology 96, 453.

10. Space and facilities available (when elsewhere than item 2 indicates, state location):

This research will be conducted at the Laboratory of Human Reproduction and Reproductive Biology, Harvard Medical School, 45 Shattuck Street, Boston, Massachusetts. We have enough laboratory space (1 x 460 sq. ft; 2 x 230 sq ft) and office space to conduct this research. Besides these spaces we share one instrument room where 3 refrigerated centrifuges, 1 ultracentrifuge, 1 scintillation counter, 1 gamma counter, 1 lyophilizer, 1 gas liquid chromatograph and 1 spectrophotometer are available for us to use. We also have sufficient space and cages to house up to 360 rats at one time, which is more than adequate for the proposed research. Other items of major equipment are fraction collectors, pH meters, balances, microscopes, electrophoretic apparatus, ovens, freezers and refrigerators.

11. Additional facilities required:

None.

12. Biographical sketches of investigator(s) and other professional personnel (append):

13. Publications: (five most recent and pertinent of investigator(s); append list, and provide reprints if available).

12. Biographical sketches

Yoshinaga, Koji

Title: Ph.D., Associate Professor of Anatomy

Born: REDACTED Sex: Male

Nationality: REDACTED

Education:		Scientific Field
B.S.	Univ. of Tokyo, Japan	Animal Physiology
M.D.	" " " "	" "
REDACTED Ph.D.	" " " "	" "
	Worcester Fdn. Exp. Biol. Shrewsbury, Mass. Postdoctoral Training	Reprod. Physiol

Honors: Awardee, Population Council Fellowship 1962-63, 1964-65.

Awardee, Lalor Found. Fellowship 1965-1966 for study at Cambridge University, Cambridge, England.

Major Research Interest: Endocrinology of female reproduction

Role in Proposed Project: Principal Investigator

Research and/or Professional Experience:

Associate Professor of Anatomy (full-time) Harvard Medical School, Boston, Mass. 7/1/72 - present.

Research, supervision of postdoctoral fellows and teaching histology laboratory to medical and dental students at Harvard Medical School. Research projects: Ovo-implantation and ovarian function.

Assistant Professor of Anatomy (full-time) Harvard Medical School, Boston, Mass. 7/1/69 - 6/30/72.

Research and teaching activities, same as above.

Research Associate in Anatomy (full-time) Harvard Medical School, Boston, Mass. 2/1/69 - 6/30/69.

Research on the same projects.

1003546114

Staff Scientist (full-time), The Worcester Foundation for Experimental Biology, Shrewsbury, Mass. 12/1/66 - 1/31/69.

Research on female reproduction. Projects: estrogen secretion by the rat ovary, uterine sensitivity and ovo-implantation.

Teaching staff of the Training Program in the Physiology of Reproduction for postdoctoral fellows.

Visiting Scientist (full-time), Agricultural Research Council, Unit of Reproductive Physiology & Biochemistry, University of Cambridge, Cambridge, England. 11/1/64 - 11/30/66.

Research Projects: Hormonal requirement for ovo-implantation, steroid hormone determination in the ovarian venous blood in the rat.

Staff Scientist (full-time), The Worcester Foundation for Experimental Biology, Shrewsbury, Mass. 1/15/63 - 10/31/64.

Research Project: Stimulatory effect of 3'5'-cyclic AMP and analogues on the synthesis of protein and phospholipids in the rat uterus.

Trainee in the Training Program in the Physiology of Reproduction (full-time), The Worcester Foundation for Experimental Biology, Shrewsbury, Mass. 1/15/61 - 1/14/63.

Training in the physiology of reproduction in general, local action of estrogen on the uterus.

Research Fellow (full-time), University of Tokyo, Tokyo, Japan. 4/1/60 - 12/31/60.
Research Project: Delayed implantation in lactating rats. Parturition of superfetation rats.

13. Five publications pertinent to the proposed work;

1) Yoshinaga, K. and C.E. Adams (1966) Endocrine aspects of egg implantation in the rat. J. Reprod. Fert. 12, 583. (relevant to egg transfer technique).

2) Yoshinaga, K. and R.O. Greep (1971) Local inhibition of ovo-implantation in the rat. Endocrinology 88, 627. (describes ovarian hormone regulation of uterine receptivity for ovum implantation)

3) Yoshinaga, K. and J.J. Ford (1974) Luteotrophic complex in lactating rats. In "Gonadotropins and gonadal function" Editor:N.R. Moudgal, Acad. Press, New York. p.260. (gonadotrophin control of ovarian steroid secretion)

4) J.J. Ford and K. Yoshinaga (1975) The role of prolactin in the luteotrophic process of lactating rats. Endocrinology 96, 335. (drug effects on LH and prolactin and ovarian progestin secretion)

5) M. Takahashi, J.J. Ford, K. Yoshinaga and R.O. Greep (1975) Effects of cervical stimulation and anti-LH releasing hormone serum on LH releasing hormone content in the hypothalamus. Endocrinology 96, 453. (method for GnRH measurement in the hypothalamus; also describes measurement of LH and ovarian steroids)

4.

14. First year budget:

A. Salaries (give names or state "to be recruited") Professional (give % time of investigator(s) even if no salary requested)	% time	Amount
Koji Yoshinaga	40%	REDACTED
(includes 16% fringe benefits)		
Technical		
laboratory technician to be recruited	100%	REDACTED
Secretary Stella Nieland	25%	
(includes 14.5% fringe benefits)		
	Sub-Total for A	REDACTED

B. Consumable supplies (by major categories)	
Rats, purchase & maintenance	1,300
Isotopes and chemicals	1,500
Glassware and plastic disposables for hormone assay	1,000
Maintenance service contract for scintillation counter	500
Sub-Total for B	4,300

C. Other expenses (itemize)	
Travel to scientific meetings	600
Publication costs	500
Sub-Total for C	1,100
Running Total of A + B + C	28,800

D. Permanent equipment (itemize)

Sub-Total for D	0
E. Indirect costs (15% of A+B+C)	4,320
Total request	$ 33,120

15. Estimated future requirements:

	Salaries	Consumable Suppl.	Other Expenses	Permanent Equip.	Indirect Costs	Total
Year 2	R	$4,500	$1,200	0	$4,575	$35,075
Year 3		4,500	1,300	0	4,815	36,915

100354616

16. Other sources of financial support:
List financial support from all sources, including own institution, for this and related research projects.

CURRENTLY ACTIVE

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates
Role of feto-placental unit in maintenance of pregnancy	NIH-HD-06467	$114,439	2-1-72 - 7-31/75
Decidual tissue as an endocrine gland	Milton Fund, Harvard University	2,800	7-1-75 - 6-30-76

PENDING OR PLANNED

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates
Endocrine regulation of ovum implantation	NIH HD-09006	(for 3 yr period) $187,742	9-1-75 - 8-31-78
Luteotrophic complex	NSF BM-75-19998	213,291	9-1-75 - 8-31-78
Role of decidual tissue in pregnancy	NIH	183,563	1-1-76 -12-31-78

Budget information: If other NIH and NSF grants are fully funded, % time of the principal investigator will be reduced from 40% to 20%. If the Council for Tobacco Research would agree, the 20% will be used for partial support of a post-doctoral fellow in order to keep up pace of the work.

It is understood that the investigator and institutional officers in applying for a grant have read and accept the Council's "Statement of Policy Containing Conditions and Terms Under Which Project Grants Are Made."

Principal investigator

Typed Name Koji Yoshinaga

Signature Koji Yoshinaga Date 6/27/75

Telephone 617 (Area Code) 734-3300 (Number) 2368,2291 (Extension)

Checks payable to

Harvard Medical School

Mailing address for checks c/o Business Office
Harvard Medical School
25 Shattuck Street
Boston, Mass 02115

Responsible officer of institution

Typed Name Henry C. Meadow

Title Executive Secretary, Committee for Research and Development

Signature Henry C. Meadow Date

Telephone 617 (Area Code) 734-3300 (Number) 441 (Extension)

10035461 17

THE COUNCIL FOR TOBACCO RESEARCH–U.S.A., INC.

January 31, 1975

Grant Application No. 1021

PULMONARY

To: The committee comprising Drs. Jacobson, Liebow and Sommers

Subject: Giles F. Filley, M.D., Webb-Waring Lung Institute, Denver
New application No. 1021
"Human Pulmonary Surfactant Function in Situ"

History

Application No. 1021 was handled by the Executive Committee as Case No. 300.

Request

This application requests $43,522 for the first year of a three year program.

Documents submitted (attached)

1. Application dated January 27, 1975 (25 pages, including C.V.'s of Drs. Filley, Paul and Newman, and G. Wayne Silvers).

2. Human subject consent form.

D.S.

DS:wg
Encl.

1003546120

1621

THE COUNCIL FOR TOBACCO RESEARCH–U.S.A., INC.
110 EAST 59TH STREET
NEW YORK, N. Y. 10022
(212) 421-8885

Application for Research Grant
(Use extra pages as needed)

Date: 1/27/75

1. Principal Investigator (give title and degrees):

Giles F. Filley, M.D.
Professor of Medicine

2. Institution & address:

Webb-Waring Lung Institute
University of Colorado Medical Center
4200 East 9th Avenue
Denver, Colorado 80220

3. Department(s) where research will be done or collaboration provided:

Department of Medicine
and
Webb-Waring Lung Institute

4. Short title of study:

Human Pulmonary Surfactant Function In Situ

5. Proposed starting date: 7/1/75

6. Estimated time to complete: 3 years

7. Brief description of specific research aims:

These will be achieved by applying three disciplines (pathology, physiology and physical chemistry) to surfactant in situ.

1) To determine the post-mortem distribution of the alveolar lining layer in healthy and diseased lungs of cigarette smokers using special fixation methods.

2) To determine the functions of surfactant in these lungs by postmortem physiological studies of airway closure, acinar clearance and alveolar collapse.

3) To determine the physical properties (especially wettability and adhesiveness) of pulmonary surfactant on dissected lung tissues in comparison to the properties on synthetic substrates.

7a. Background information relevant to the objectives and working hypothesis:

The fact that surface phenomena contribute to lung retractive force, though reported by von Neergaard in 1929, was not generally appreciated till the direct demonstration of Pattle (1955) that the material lining alveoli had special properties. By squeezing bubbles from slices of lung at postmortem he obtained enough material to deduce one of its important physical properties--that its surface tension is "nearly zero" in vitro, that is to say, as the lining of a

bubble outside the lung. His method of sampling the alveolar lining layer (as he called it) has been subsequently replaced in most laboratories by lung mincing or lung lavage methods, and the material so obtained has been subjected to extensive chemical analyses and surface balance studies. Valuable as these in vitro studies are, their indirect nature must be recognized and they must be correlated with the behavior of the lining layer in situ.

Because of the delicacy and inaccessibility of alveoli in the living lung very few direct physiological measurements have been made of alveolar wall deformation. In vivo observation of the lung surface (Wagner 1970) has not yielded quantitative information as to how this surface changes geometrically and our knowledge is largely limited to the results of measurements on quick frozen lung (Klingele and Staub 1970, Glazier 1967, Hughes et al 1970) or specially fixed tissue (Weibel and co-workers 1968, 1972, 1973). The latter have shown that alveoli are more polyhedral in shape than spherical, and that, at least in rat lungs fixed at three levels of inflation, the alveoli, rather than stretching with inflation, expand in a more complex manner perhaps best described as an "unpleating." The surface lining layer furthermore, rather than being a monolayer on a smooth surface, is distributed as sheets of variable thickness smoothing out tissue irregularities and as "pools of lining layer in crevasses." These authors have confirmed the finding of Klingele and Staub (1970) that below the physiologic range the alveoli "fold up from side to side as an accordion or concertina folds rather than by uniform decrease in all directions until they disappear as implicit in lung models based on the soap bubble analogy." Finally they have shown again in rat lungs, that the mean radius of curvature of expanded alveoli is about twice as large as in contracted alveoli and that the surface area-to-volume ratio was nearly constant instead of being inversely proportional to the radius of curvature as required by a spherical alveolar model.

How small airways 0.3 to 2.0 mm in diameter (alveolar ducts to 9th order bronchi) become narrowed and "close" is of vital importance to pulmonary physiology and chest medicine. Because of their location most investigations of small airway function have been indirect and their behavior has only recently begun to be understood (Macklem 1972). Direct intraluminal pressure measurements via tiny catheters have, thru the work of Macklem and his group (1965, 1967), revolutionized modern ideas of the mechanisms of airway obstruction and radically changed the meaning of standard ventilatory function tests (Mead 1970).

Excised lung tissue from experimental animals, meticulously dissected and studied by physiologic methods (Murtagh et al 1971, Menkes et al 1971, Hughes et al 1972), are being used to determine the relative contributions of intrinsic forces in airway walls, the luminal surface tension and the tissue forces of surrounding lung parenchyma. The recent review of Mead (1973) indicates the importance of these direct approaches to small airway function.

A recent direct attack on the problem of airway closure has been interpreted by the traditional concepts of how pulmonary surface forces operate, which we have examined above. Thus Macklem, Proctor and Hogg (1970) after recording

P-V curves of air-filled dissected cat bronchioles before and after flushing with 2% Tween or dog lung extracts concluded "that surfactant lines and stabilizes bronchiles protecting against excessive radius changes with lung volume and air trapping. Their studies contradicted in some ways the finding of Cavagna et al (1967) that all airways are open at a transpulmonary pressure of zero, but the discrepancies were accounted for by "geometric factors" associated with distortion of airways consequent to the dissection procedure. The discrepancy between the results and those of others has been repeatedly referred to (Burger and Macklem 1968, Macklem et al 1970, Macklem 1971, Ingram et al 1974) and must be resolved.

Small airways, however, have many potential functions other than "staying open." A small airway's ability to convey liquid material originating from alveoli and, in particular to act as a conduit for the removal of toxic material from alveolar walls may be equally important. Unfortunately, although we know a fair amount about how ciliated airways are kept clear of unwanted material (Litt 1974), it is much less certain how alveolar tissue and small airway clearance mechanisms operate (Gross 1964, Staub 1966, Mendenhall 1972, Tucker et al 1973). New concepts are being developed to understand how cilia-free alveolar ducts and respiratory bronchiles clear themselves of noxious material. The importance of knowing whether or not surface active material is normally present and how it functions in the small airway lies in the possibility that changes in such material could well be produced by the inhalation of toxic substances. Thus DuBois and Rogers (1968) have concluded that the smaller bronchi are the most vulnerable sites for damage from inhaled particles. If surfactant is present in small bronchi, it can be shown (Sec. 9F) that toxic materials would be propelled toward the mouth by surface tension forces (i.e., without cough or ciliary action). If surfactant is absent or defective in the small airways of patients with emphysema, an obvious patho-genetic argument for the development of alveolar lesions early in this disease could be developed. Kilburn (1974) has presented evidence that certain small airways (those just at the junction of true alveolar tissue and the conduit bronchioles cleared by ciliary mechanisms), are a "no-man's land" as regards clearance, i.e. that they are in a region "cleared either way." It is clearly important to determine whether or not surface active material is present in such small airways and if it is capable of providing a clearance mechanism.

Finally, R.V. Ebert and M.J. Terracio have very recently (1975) shown that fresh surgical lung specimens from smokers show a loss of clara cells which may secrete a special sol (a strongly surface active colloid) in which cilia beat to move the overlying mucous gel. As Ebert says: "Unfortunately there is little direct evidence as to the nature of the surface coating of the bronchioles." Furthermore he emphasizes that when fixation via bronchi is used "the surface material is readily removed"--a point we consider in detail below (9.D.) The next page shows the delicacy of the surface we are considering as reproduced from page 9 of Ebert's paper.

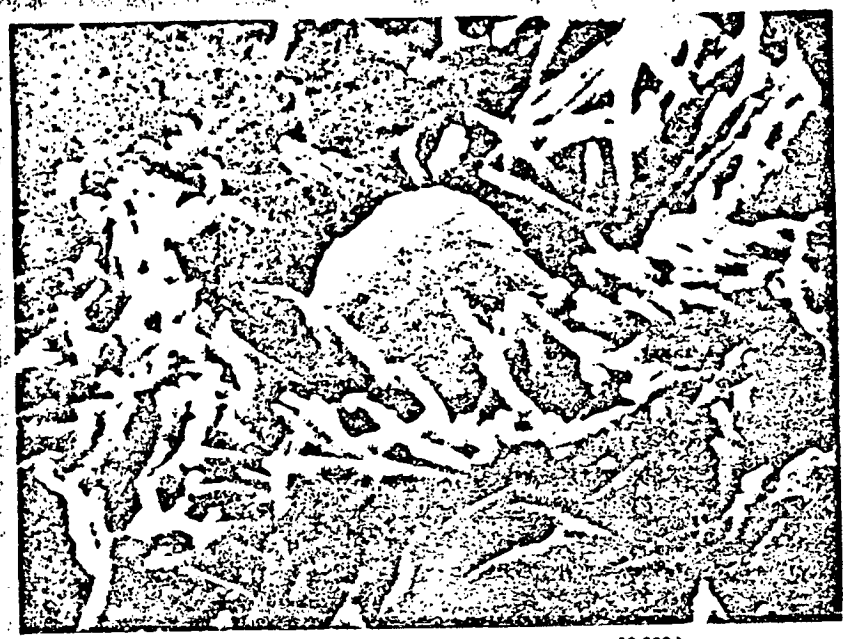

Fig. 5. A Clara cell surrounded by cilia. (Original magnification: × 10,000.)

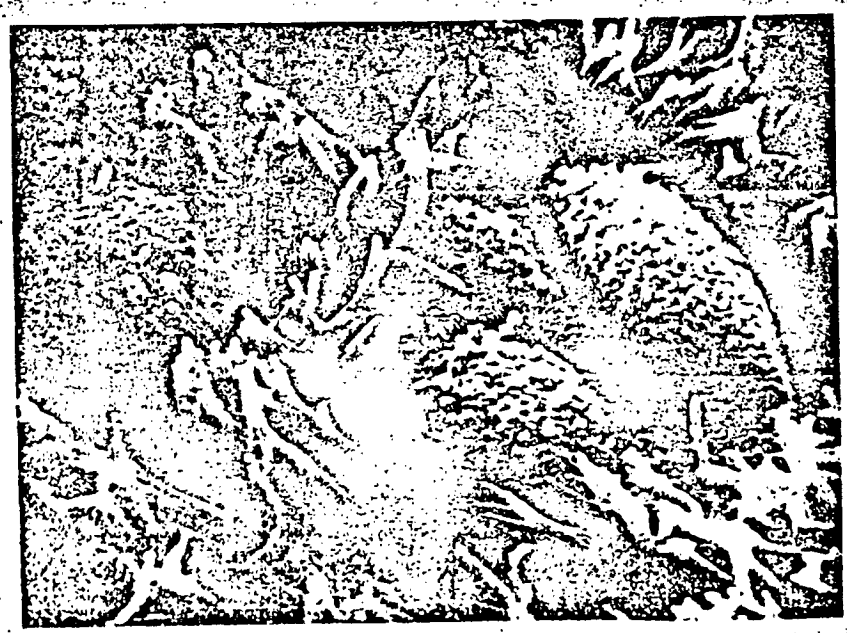

Fig. 6. Epithelium of a bronchiole 225 μm in diameter demonstrating transitional cells and ciliated cells. (Original magnification: × 5,250.)

B. Brief statement of working hypothesis:

Although pulmonary surfactant is important in expanding the lungs of the newborn, its role in adult lungs, where it is continually produced is not understood. Despite the widespread belief that surfactant, by varying the air-alveolar surface tension, prevents alveoli from collapsing on expiration and from overstretching on inspiration, atelectatic or emphysematous adult human lungs have not been shown to yield abnormal surfactant on pulmonary lavage or by analysis of lung extracts. Our hypothesis is that surfactant provides an important protective function and that maldistribution of surfactant allows cigarette smoke to narrow airways, promote alveolar pleating and damage Type I alveolar cells.

Failure of surfactant to form a ubiquitous layer and convey deposited material from the acinus would expose Type I cell walls to direct and prolonged effects of inhaled substances. The remarkable fact that the vast majority of cigarette smokers do not acquire emphysema suggests that most human lungs are as efficient in protecting their alveoli as tears are in protecting the eyes. To study the chemistry of tears in vitro would reveal very little of their protective function. Thus our insistence that bronchial and alveolar surfaces be studied in situ.

9. Details of experimental design and procedures (append extra pages as necessary)

A. Sources of human lungs, temporal effects and pathology

Human lungs will be obtained from the autopsy service of the Department of Pathology of the University of Colorado Medical Center. A special source will be cadaver transplant donors (kidney and liver) whose usually normal lungs will be made available by the Department of Medicine (Dr. T.L. Petty) and the Division of Biochemistry of the Webb-Waring Lung Institute (Dr. O.K. Reiss).

There are two potential sources of error which must be considered in regard to studies of pulmonary surfactant when the source of the lungs is human beings at autopsy, namely, the time postmortem and the method of sampling surface-active lung fluids. Avery and Mead (1959) reported that in dog lungs there was no significant difference in their surfactant assay results if lungs were studied immediately after death or after refrigeration or freezing for as long as six days. Gruenwald et al (1962) showed that adult human lungs frozen at -20° C and examined six weeks after autopsy were considered satisfactory. Reynolds et al (1965) demonstrated that in lungs of newborn infants obtained within 24 hours of death and stored at -4° to -10° C and then studied within three days, there was no change when surfactant assay was re-examined 12 months after storage. However, the effect of time postmortem on pressure-volume measurements is still not settled. Faridy et al (1966) have shown that dog lobes do not show significant differences in pressure-volume measurements if the lobes were at low temperature for five days, yet Bachofen et al (1970) showed slight changes in pressure-volume curves of cat lungs after three hours after death. Accordingly, we will have to determine the time postmortem effects of the human lungs with which we will be working. We will do this by carrying out repeated pressure-volume and surfactant assay measurements.

Facilities of the Division of Pathology of this Institute will be used to assess the lungs for their normality and to determine the nature of any pathological processes in the tissue. Smoking histories will be documented when available.

B. Surfactant isolation

For the experiments in Sec. 9G, we will isolate surfactant material by saline lavage (0.9% NaCl) of human postmortem lungs. Serial lavages will be pooled and

centrifuged at 850 xg to remove alveolar macrophages and other cellular debris. The supernatant will be spun at 10,000 xg to concentrate the surface active material into a pellet. The pellet, which will be resuspended in 0.9% NaCl, is referred to as whole or crude surfactant preparation.

The isolation procedure proposed here is based on experience in this laboratory with rabbit, pig and sheep lung surfactant. Each step will therefore have to be checked for its applicability to the isolation of human material, and this is currently under way in the laboratory of Dr. O.K. Reiss at this Institute. When it becomes desirable to study the purified lipid and protein fractions, such material will be obtained by the zonal density centrifugation methods worked out in this laboratory (Reiss 1970, Gil and Reiss 1973).

The use of micropipettes for direct sampling of surfactant from the alveolar surface and micromethods for the study of its physicochemical properties has been reported by Reifenrath and Zimmermann (1974). Since direct methods have proven decisive in other areas of this field these micromethods seem worth exploring. Staub (1974) has reported the astounding fact that in a related field of tremendous clinical importance (pulmonary edema physiology) "No one except Nitta (1973) has ever obtained direct alveolar fluid." Direct microscmpling methods of surfactant isolation will be used as micromethods of physical and chemical study develop (second and third year).

C. In situ P-V methods

Pressure-volume measurements

Whole human lungs will be ventilated with air and with liquids to determine their quasi-static P-V characteristics; in addition, lobes, segments and sub-segments will be dissected from whole lungs and similarly ventilated. Lungs and portions of lungs will be placed in a volume-displacement plethysmograph similar to the one described by Bachofen et al (1970). This apparatus allows air and liquid pressure-volume curves as well as degassing to be performed without removing the tissue from the plethysmograph.

Our experience with degassing human lungs in the manner often done with animal lungs (Johnson et al 1964), by placing them in a vacuum jar and evacuating the jar until water vaporization and recompression, often resulted in tears and rupture of the parenchyma. This we felt was due to collapse of the small airways trapping gas in the alveoli and eventual rupture of the parenchyma on further evacuation of the vacuum jar. This is overcome by the technique of von Neergaard (1929). A water valve set at 8-10 cm H_2O is inserted between the cannula to the lung and the plethysmograph. With the evacuation pump operating simultaneously both on the bronchus and the space between the lung and plethysmograph, the pressure outside the lung is maintained to about 8-10 cm water lower than in the bronchus. The alveoli thus are in free communication with the bronchial tree until full evacuation, i.e., water vapor pressure, is reached. This will allow filling with air or liquid without any walls being opposed or "stuck together," which filling may well give different results than when collapse is allowed to occur as in conventional degassing. The two techniques will be compared.

An air pressure-volume curve will be done after degassing. The lungs will be inflated to +20 to 22 cm water and deflated to zero pressure three times to standardize volume history. Beginning with the fourth inflation, volume will be

monitored at 1 cm of water pressure increments until +20 cm of water is reached. Thirty seconds will be allowed between measurements for equilibration of pressure. The same procedure will be followed on the deflation limb of the pressure-volume curve.

As indicated above we will dissect, from normal and pathologic human lungs, portions for direct study of mechanical and other properties connected with surface activity. Isolation of the superior segment of the right lower lobe has already been accomplished (see Fig. 1) along with cannulation of the superior segmental bronchus, B_6, (Bloomer et al 1960). The problem of leaks has been dealt with by gluing the edges of the cut surface to a glass plate with contact cement (Permabond 102, Pearl Chemical Co., Tokyo). Dissection of subsegments of the superior segments of both the right lower and left lower lobes will be carried out with the help of a Magnifying Loupe, Iris forceps, Micro Jameson Scissors and similar surgical equipment (already purchased on the advice of Dr. Melvin Newman). By making use of many representative portions of human lungs we hope to obtain the maximum information possible from a single human lung, especially when the experiments involve ventilation and fixation at various levels of inflation with expensive materials.

D. In situ surfactant distribution

The very recent report of Callas (1974) shows that even after the painstaking work of Gil and Weibel (1969-70) "all published reports indicate failure to demonstrate surfactant as a layer lining the entire alveolar surface." Callas has modified Weibel and Gil's method by attempting to "plaster" the surfactant to the alveolar cells by filling the lungs with agar. In doing this, however, he is compelled to force the agar into a completely collapsed lung (since the air in an inflated lung would prevent the agar from penetrating to alveolar surfaces). Despite the use of his elaborate method, Callas makes the remarkable statement that "no alveoli were found to be completely lined with surfactant."

We believe that no method which introduces a liquid into a lung (especially one that is collapsed) can reveal the normal in situ distribution of surfactant. We therefore propose to use the special degassing technique worked out by von Neergaard (see Sec. 9C) for another purpose. The lungs, instead of being degassed in the usual way in which alveolar surfaces are allowed to fold up, collapse and touch each other (which, we think, must considerably change the distribution of surfactant), are degassed by removal of air molecules without change of lung volume. After this has been accomplished fixation by vaporized or aerosolized glutaraldehyde will be carried out. This method will imitate, to some extent, the formalin steam method of Weibel and Vidone (1961), but will be modified to insure that the lung neither gains nor loses water (Wright et al 1974). In this way the alveolar lining layer will be fixed in situ, its distribution unaffected by processing and fixation artefacts--probably for the first time.

After glutaraldehyde fixation both conventional and electron microscopy methods will be used. Briefly, we will use the ultravioled microscopy technique of Balande and Klause (1964), who concluded that the alveolar lining layer was revealed in guinea pig lungs by fluorescent lines less than one micron thick. The alveolar lining layer of human infants has also been observed using this technique (DeSa 1965). The same tissue fixed in the previously described manner

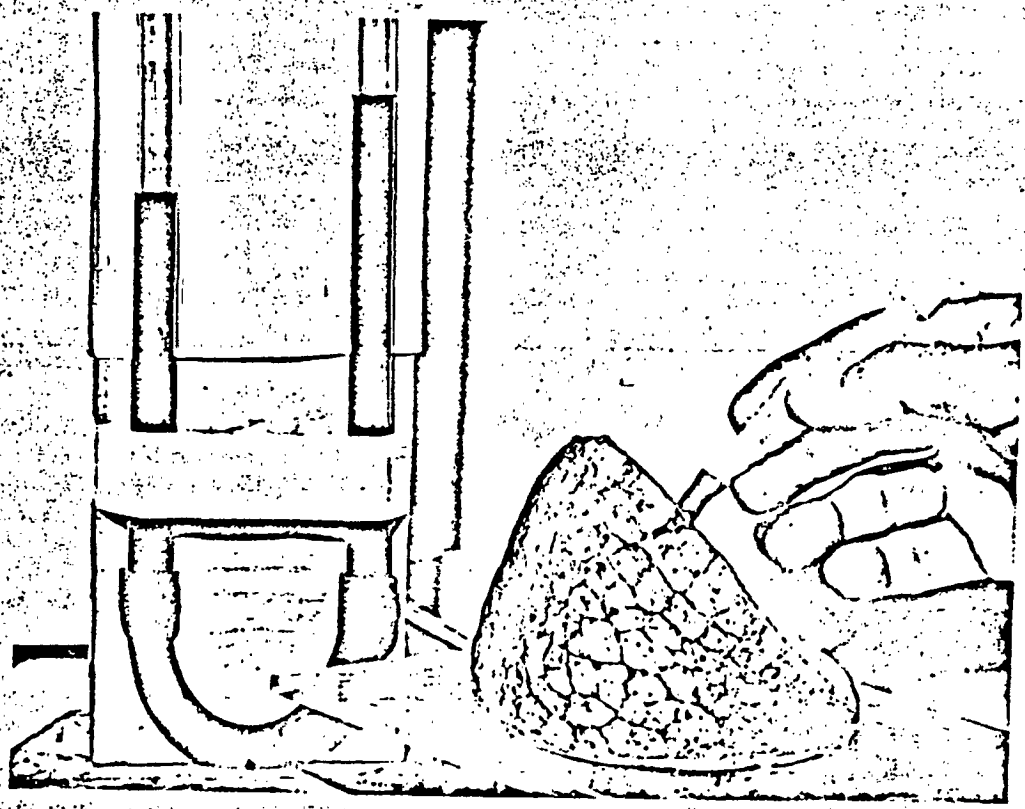

Fig. 1. Superior segment of the right lower lobe of a normal human lung. The cut surface of the posterior basal lobe is resting on a glass plate and glued at the edges. The superior segmental bronchus is cannulated for P-V measurements.

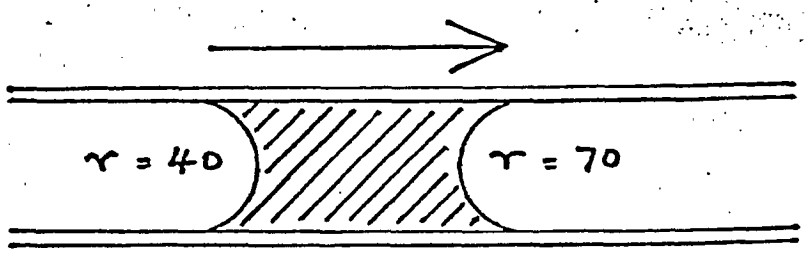

Fig. 2. The fluid is "pulled" in the direction of the arrow by the greater surface energy at the right meniscus. If the right meniscus is facing the trachea or the left the alveoli, surface energies could have a definite role in clearing material from small airways (Schwartz et al., 1964).

may be used for electron microscopy. Post-fixation methods as described by Callas (1974) will be used in conjunction with the EM facilities of the Division of Cell Biology of this Institute.

E. Methods for the direct study of airway closure mechanisms in the lungs of smokers and non-smokers

Lungs which have had pressure volume curves constructed, as described above, will be dissected to remove a section of the bronchial airways of 2-3 mm in diameter and less. (Surgical advice and assistance will be given to us by Dr. Melvin Newman.) The largest airway will be cannulated while the rest will be tied off with suture. The specimen will be placed in a small plethysmograph equipped with an optical window for microscopic observation similar to that described by Martin and Proctor (1958). The cannulae will be 10 and 20 microliter pipettes, which will be calibrated previously with a Hamilton microliter syringe. A small droplet of water will be placed within the pipette and will serve to mark the volume of air entering the airways. Pressure will be measured by use of a leveling bulb, on a Statham strain-gauge pressure transducer (P23Gb). Thirty second intervals will be allowed for equilibration. A minimum of four complete inflation and deflation pressure-volume curves will be done to check on reproducibility. A similar procedure will be followed when pressure-volume curves of the specimen in various liquids are to be obtained. The plethysmograph will be completely filled with liquid as well as the leveling bulb so that now pressure readings will be obtained by reading the difference between the menisci of the pipette and leveling bulb. The pressures at which the airways open and close will be recorded. From these data we can determine if the airways close at the same or at different pressures in the air filled lung, indicating if surface forces influence small airway closure, and relating these findings to the smoking habits of the patient in life.

Direct microscopy will also be used to determine the relative roles of surface intrinsic and extrinsic forces controlling the behavior of the 0.3-2.0 mm airways of human lobes. Lobes of lungs obtained at surgery or autopsy will have the tracheobronchial tree exposed by dissection with the aid of a dissecting microscope. By cannulating the airways with a blunt needle, pressure-volume curves can be achieved with a microliter syringe and a small volume displacement pressure transducer as described by Macklem et al (1970), the pressures at which different sized airways open and close can be measured and noted with the dissecting microscope. This procedure can be performed by using air, saline, or by flushing with a substance with a known surface tension such as Tween, reflushing with air and repeating the measurements using air. In this manner we can change the surface tension from that normally found in airways to that approaching the tension of the fluid just flushed. Because the geometry and mechanics of dissected airways differs from that of bronchi in the intact lung, we will, in the above methods, be relying on the comparative behavior of airways lined with different materials. If initial volumes and pressures are held constant for each experiment, the differences between P-V relationships should be informative even though the "extrinsic factors" due to lung parenchyma are different than in life (Murtagh et al 1971, Hughes et al 1972, Hughes et al 1973).

To determine the geometry of closure of airways (lined with naturally occurring surfactant or other materials) as lung tissue volume is reduced, the quantitative methods of Gil (1971) will be used. The bronchi and pulmonary artery supplying the

lobes or segments of human lungs will be cannulated. By controlling the airway pressure, the lung volume and the flow of fixative, the lungs will be fixed at several points along both inflation and deflation limbs of the P-V curve. Fixation of lungs and appropriate sectioning and microscopic study of the small airways will reveal the morphologic changes they undergo as they "close" during deflation and "open" from a state of closure and should clarify the nature of airway P-V hysteresis (Mead 1973).

To localize portions of airways of particular interest we will, before fixation and/or sectioning, use the facilities of the Magnification Unit in the Department of Radiology. Here a FAXITRON X-ray apparatus (Hewlett-Packard) will enable us to use low energy X-rays. The device has an ultrafine focal spot (0.5 mm) and a beryllium window. Thin pieces of lung will be examined in order to minimize distortion from overlapping of airways. The advantage of this technique is that it allows us to visualize airways as small as 200 μ in diameter and to avoid the use of contrast media after the manner of Murtagh et al (1971).

F. Method for the direct study of airway clearance mechanisms

As Macklem has postulated (1971), based on direct observation of dissected bronchioles, fluid in small airways could form two hemispherical menisci in vivo, one facing the trachea and the other facing the alveoli. His analysis was restricted to considerations of airway stability. However, such fluid menisci are known to have properties which affect fluid movement and which may be important as part of a lung clearance mechanism.

Well-developed formulations (Davies and Rideal 1961, Schwartz et al 1964) relate the movement of a liquid through air-filled horizontal tubes to the surface tension of the liquid and the contact angle between the liquid and tube wall surface. Both theoretically and experimentally, liquid segments of the type visualized by Macklem move through capillary tubes in directions and at rates determined by the difference between the surface tensions at the two menisci. (See Fig. 2). Using the methods discussed under Sec. 9E for dissecting out and studying the bronchi of human lungs, we will investigate, by direct microscopic observation, the effect of naturally present and foreign surface-active materials to the importance of locally varying surface tensions in clearing material from alveolar tissue.

G. Wettability and adhesive properties of pulmonary surfactant on dissected lung tissues and on synthetic materials

As a result of our independent theoretical development, we have concluded that there are several "surface tensions" of importance in the lung. We visualize the surfactant system of the lung as a lining layer (ll) of surfactant "sandwiched" between the air (a) phase and the tissue hypophase (h). The tensions (γ) between the lining layer and air or tissue are given by $\gamma_{ll,a}$ and $\gamma_{h,ll}$ respectively; at places where the tissue is not covered with surfactant the tension is $\gamma_{h,a}$. The relationship among the tensions is given by the equation of Young (1805):

$$\gamma_{h,a} - \gamma_{h,ll} = \gamma_{ll,a} \cos\theta$$

where θ is the contact angle between the surfactant layer and the tissue. $\theta = 0$ is the condition for complete spreading of surfactant on the tissue.

The purpose of the wetting experiments is to infer the contribution of the tensions at the tissue-air and tissue-surfactant interfaces to the total surface force in the lung, and to determine the spreading characteristics of surfactant on normal and diseased tissue. This is important because, for example, cigarette smoke may alter the tissue while the surfactant appears "normal."

Thin slices of alveolar tissue or small airways, the cut surfaces on which the wettability and adhesion of surfactant will be measured, will be obtained with a tissue slicer or by hand dissection. Chemical fixatives will of course have to be avoided because of possible alteration of the surface properties of the tissue. For the present experiments we will use an automated Wilhelmy film balance of conventional design. Details of construction for the particular balance to be employed have been previously described (Dreher and Wilson 1970). The balance has since been modified to measure γ-A isotherms over a wide range of frequency and to interface with a H-P 7001 x-y recorder. The balance will be suitably mounted to eliminate the effect of vibrations, and cleanliness of chemicals, glassware and surfaces will be in accordance with accepted procedures (Gaines 1966).

Young's equation may be rewritten as $\gamma_{adh} = \gamma_{11,a} \cos\theta$, where $\gamma_{adh} = \gamma_{h,a} - \gamma_{h,11}$ is the adhesion tension. The completeness and reversibility of spreading will be determined by the wetting balance method of Guastalla (1957). If $\theta \neq 0$ then a Wilhelmy plate of platinum measures $\gamma_{11,a} \cos\theta$. Thus, if a plate is constructed of a thin slice of lung tissue (or two slices of tissue with a thin piece of glass or metal sandwiched between them for stability) and the force $\gamma_{11,a}\cos\theta$ transmitted to this tissue plate by a spread film of surfactant on water is measured, then the simultaneous measurement of $\gamma_{11,a}$ with a roughened ($\cos\theta=1$) platinum or glass plate in another part of the film will yield the value of $\cos\theta$ as well as γ_{adh}. In this way the validity of the assumption that surface forces at the air-water interface are similar to the forces on the surface of the lung can be tested in a more direct manner than has heretofore been proposed. For example, if the advancing and receding contact angles are widely different, the γ-Area hysteresis loop traced by the force on the tissue plate would reflect the importance of wettability to the P-V loops in air filled lungs. Similarly, by substituting a liquid for air, the γ-Area loops so obtained should reflect conditions at the interface of a liquid filled lung.

To estimate $\gamma_{h,a}$, the $\cos\theta$ of droplets of low energy liquids (fluorocarbons) of known surface tension ($\gamma_{f,a}$) will be measured on lung tissue which has been rinsed with saline. $\gamma_{h,a}$ may be estimated by application of the equation of Good (1964) and Fowkes (1962):

$$\gamma_{h,a} = \gamma_{f,a} (1 + \cos\theta)^2 / 4\phi^2 \gamma_{f,a}$$

where ϕ is a numerical constant. Lung tissue strips will be mounted horizontally in a small plexiglas chamber with ports for evacuation or saturation with gas. The θ's will be measured with a cathetometer telescope and a gionometer eyepiece. The magnitude of $\gamma_{h,a}$ and $\gamma_{h,11}$ (which is now known from $\gamma_{h,a}$ and the previously measured γ_{adh}) relative to $\gamma_{11,a}$ will reflect the importance of the tissue stress in the total retractive force in the lung.

Methods of measuring the adhesive force between similar or dissimilar surfaces coated with a liquid film have been known for many years (Budgett 1912). These forces are often quite high, thus it is worthwhile to determine if reductions in the adhesive force can be effected by pulmonary surfactant. This problem will be approached by using a duNouy tensiometer to measure the vertical force required to separate plates of synthetic materials (glass, metal, teflon) or slices of lung tissue coated with surfactant. Since lung surfactant is known to be viscoelastic (Kott et al 1974), the results will depend on the rate of separation of the surfaces. Initially the experiments will be confined to quasi-static conditions. By comparing the work required to separate lung tissue in air with that required in saline, the idea is to show that the known reduction in retractive force in liquid filled lungs relative to air filled lungs can be explained by an alternate mechanism of surfactant operation. For example differences in the air and saline work of adhesion would indicate that surfactant could produce the difference seen between air and liquid P-V curves without significant changes in lung area by stretching of the alveolar surface, but rather by a simple unfolding of alveolar pleats.

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Mead J: Respiration: pulmonary mechanics. Ann Rev Physiol 35: 169, 1973.

Mendenhall RM: Surface spreading of lung alveolar surfactant. Resp Physiol 16: 175, 1972.

Menkes A, Gardiner A, Gamsu G, Lampert J, Macklem PT: Influence of surface forces on collateral ventilation. J Appl Physiol 31: 544, 1971.

Murtagh PS, Proctor DF, Permutt S, Kelly BL, Evering ES: Bronchial mechanics in excised dog lobes. J Appl Physiol 31: 403, 1971.

Nitta S, Staub NC: Lung fluids in acute ammonium chloride toxicity in cats and guinea pigs. Am J Physiol 224: 613, 1974.

Pattle RE: Surface lining of lung alveoli. Physiol Rev 45: 48, 1965.

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Reynolds EDR, Orzalesi MM, Matoyama ED, Craig JM, Cook CD: Surface properties of saline extracts from lungs of newborn infants. Acta Paed Scand 54: 511, 1965.

Schwartz AM, Rader CA, Huey E: Resistance to flow in capillary systems of positive contact angle. Adv Chem Ser 43: ACS Publications, Washington DC, 250, 1964.

Slama H, Schoedel W, Hansen E: Lung surfactant: film kinetics at the surface of an air bubble during prolonged oscillation of its volume. Respir Physiol 19: 233, 1973.

Staub NC: The "liquid veins" of the lung. Abstr, Physiologist 9: 294, 1966.

Staub NC: Pulmonary edema. Physiol Rev 54: 678, 1974.

Tucker AD, Wyatt JH, Undery D: Clearance of inhaled particles from alveoli by normal interstitial drainage pathways. J Appl Physiol 35: 719, 1973.

von Neergaard K: Neue Auffasungen über einen Grundbegriff der Atemmechanik. Die Retraktionskraft der Lunge, Abhängig von der Oberflächenspannung in den Alveolen Z Ges Expl Med 66: 373, 1929.

Wagner WW Jr: Pulmonary microcirculatory observations in vivo under physiological conditions. J Appl Physiol 26: 375, 1970.

Weibel ER, Vidone RA: Fixation of the lung by formalin steam in a controlled state of air inflation. Am Rev Resp Dis 84: 856, 1961.

Weibel ER, Gil J: Electron microscopic demonstration of an extracellular duplex lining layer of alveoli. Respir Physiol 4: 42, 1968.

Weibel ER: Morphological basis of alveolar capillary gas exchange. Physiol Rev 53: 419, 1973.

Weibel ER, Untersee P, Gil J, Zulauf M: Morphometric estimation of pulmonary diffusion capacity. VI. Effect of varying positive pressure inflation of air spaces. Respir Physiol 18: 285, 1973.

Williams R: Computer analysis and model of the properties of pulmonary surfactant: effects of nitrogen dioxide. Ph.D. Dissertation, Pennsylvania State Univ, University Microfilms, Ann Arbor, Mich, 1970.

Wright BM, Slavin G, Kreel L, Callan C, Sandin B: Postmortem inflation and fixation of human lungs. Thorax 29: 189, 1974.

Young T: On the cohesion of liquids. Phil Trans 95: 65, 82, 1805.

Ebert VR, Terracio MJ: The bronchiolar epithelium in cigarette smokers. Observations with the scanning electron microscope. Am Rev Resp Dis 111: 4, 1975.

10. Space and facilities available (when elsewhere than item 2 indicates, state location):

A 600-square foot laboratory in the basement of the Webb-Waring Lung Institute (W-WLI) is available for the postmortem lung mechanics studies and for the isolated surfactant studies. A pathology laboratory equipped for inflation-fixation and cutting of lungs, preparation of histologic sections and long-term storage of fixed lung slices and histologic material is available on the fourth floor of W-WLI.

Major items of equipment availabor for this work at W-WLI include:
Electronics for Medicine IR-r recording equipment
Hewlett-Packard 7001 x-y recorder
Beckman GC2-A gas chromatograph
Electronics for Medicine DR-8 and assorted transducers
Monoghan respirator
Harvard respiration pump
Beckman Spinco Model L Ultracentrifuge
International Refrigerated Centrifuge Model PR-2
Sorvall RC2-B refrigerated centrifuge
Mettler M-5 microbalance
Automatic quartz bi-distillation unit for production of pure water
Automated Wilhelmy surface balance modified for variable frequency and temperature controlled gas studies
Collins spirometer
Phillips 300 Electron Microscope

The Department of Radiology is equipped with Faxitron X-ray facilities and auxiliary equipment.

11. Additional facilities required:

None

12. Biographical sketches of investigator(s) and other professional personnel (append):

13. Publications: (five most recent and pertinent of investigator(s); append list, and provide reprints if available).

CURRICULUM VITAE

GILES F. FILLEY, M.D.

June 1972

Birth Date:

Marital Status: REDACTED

Children: REDACTED

Education: Kent School, Kent Conn. Graduated R -- High School
Williams College, Williamstown, Mass. Graduated REDACTED
Johns Hopkins University, Baltimore, Md. Graduated REDACTED

Internship: Johns Hopkins Hospital, Baltimore, Md. 1942-1943.

Residencies: Lawrason Brown Fellow, Department of Physiology, The Edward L. Trudeau Foundation, Saranac Lake, New York 1943-1944.

Assistant in Medicine, Johns Hopkins University. 1944-1945.

Instructor in Medicine and John D. Archbold Fellow in Medicine, Johns Hopkins University. 1945-1946.

Assistant Resident Physician, Medical Ward Service, Barnes Hospital, St. Louis, Mo. 1946-1947.

Positions: Associate Physiologist, Department of Physiology, The Edward L. Trudeau Foundation, Saranac Lake, New York. 1947-1953.

Director, Department of Physiology, The Trudeau-Saranac Institute, Saranac Lake, New York. 1953-1955.

Assistant Professor of Medicine, University of Colorado School of Medicine, Denver, Colorado. 1955-1959.

Associate Professor of Medicine, University of Colorado School of Medicine, Denver, Colorado. 1959-1969.

Professor of Medicine, University of Colorado School of Medicine, Denver, Colorado. 1969-Present.

Clinical Physiologist, Chief-Division of Physiology, Webb-Waring Lung Institute, Denver, Colorado. 1955-Present.

Memberships in Societies:

REDACTED REDACTED

REDACTED

Am Col Phys

Consulting Positions: Denver General Hospital
Fitzsimons Army Hospital
Veterans Administration Hospital

100354613

RESUME

George W. Paul

REDACTED

Telephone:
(303) 394-8731 (Office)

PERSONAL

REDACTED REDACTED

RESEARCH INTERESTS

Biomedical applications of surface chemistry, structure of water and liquid solutions, membrane phenomena, thermodynamics (equilibrium and non-equilibrium), transport processes. Have reviewed about 400 papers in these areas. Long range: Teaching or research institute.

EDUCATION

University of Missouri (Columbia): B.S. R M.S. R Ph.D. R all chemical engineering. G.P.A.: R

M.S. Thesis: "Interfacial Free Energy in Binary and Ternary Systems."

Ph.D. Comprehensive Problem: "An Investigation of Solute Diffusion in Polymer Solutions."

Ph.D. Dissertation: "Interfacial Tension in the Critical Region."

EXPERIENCE

Summer 1963: U.S. Gypsum Co., Mexico, Missouri. Polymer coating technology.

Summer 1964 and 1965: Nalco Chemical Co., Chicago, Illinois. Correlation of kinetics of co-polymerization reactions.

Summer 1966 and 1967: Research Assistant, Engineering Experiment Station, University of Missouri, Columbia, Missouri. Undergraduate instruction in techniques of digital computation.

8/69 to 11/72: Standard Oil Co. (Ind.) Research Center, Tulsa, Oklahoma. Surfactants and biopolymers (polysaccharides). Evaluation of experiments on micellization, non-equilibrium adsorption, flow through porous media.

11/72 to present: Postdoctoral Research Fellow, Webb-Waring Lung Institute, University of Colorado Medical Center, Denver, Colorado. My present work is focused on understanding the physical chemistry of the lining of human lungs. The surface of mammalian lungs in general is composed of a special fluid of peculiar surface properties. It is known that some diseases result directly from disturbances in these surface properties. It is likely that even more common diseases such as emphysema involve changes in the chemistry of these substances and my work is devoted to determining these changes.

ACTIVITIES

1968, Teen Club Worker, Columbia, Missouri. Avid tennis player, reader of contemporary history.

PROFESSIONAL ORGANIZATIONS

REDACTED REDACTED REDACTED

HONORS AND AWARDS

Green Scholarship, Curators Scholarship, American Chemical Society Research Fellowship, NASA Fellowship, Omicron Delta Kappa, Pi Omicron Sigma.

REFERENCES

Professor Marc deChazal, Department of Chemical Engineering, University of Missouri, Columbia, Missouri 65201; Dr. J.T. Ryan, Department of Chemical Engineering, University of Alberta, Edmonton, Alberta, Canada; Dr. P.D. Shoemaker, Monsanto Company, 800 N. Lindbergh, St. Louis, Missouri.

Resume

G. Wayne Silvers

Born: REDACTED

Education: University of Colorado, Boulder, Colorado
B.A., Zoology, 1963.

Appointments: Research Associate, University of Colorado Medical Center, Denver, Colorado, 1972 - present.

Research Physiologist, University of Colorado Medical Center, 1967-1972

Research Technician, University of Colorado Medical Center, 1963-1967.

3e

CURRICULUM VITAE

Melvin M. Newman, M.D.

Place and Date of Birth: REDACTED

College: University of Chicago, R

Medical School: University of Chicago, R B. S. R, M. D. R

Honor Societies: Phi Beta Kappa, Alpha Omega Alpha, Sigma Xi

Rotating Internship: University of Chicago Clinics, September 1944 - June 1945

Residency Training: University of Chicago Clinics, October 1946 - July 1952
General and Thoracic Surgery

Teaching and Research Appointments: Instructor in Surgery, University of Chicago, July 1950-- June 1952

National Research Council Fellow, University of Chicago
July - October 1952 (Recalled to Active Duty, U.S. Navy)

Assistant Professor of Surgery, State University of New York
College of Medicine at Brooklyn, July 1954 - March 1956

Associate Professor, March 1956 - August 1959

Director of Surgery, National Jewish Hospital at Denver,
September 1959 to August 1968

Clinical Associate Professor of Surgery, University of Colorado,
1961 to 1968

Associate Professor of Surgery (Full-time), University of Colorado,
1968 to present

Certification: American Board of Surgery - 1951; Board of Thoracic Surgery - 1953

Military Service: Lt. J.G. MC USNR, July 1945 - August 1946
Lt. MC USNR, October 1952 - July 1954 (Chief of Thoracic Surgery,
U. S. Naval Hospital, St. Albans, N.Y., August 1953 - July 195

Marital Status: Married September 11, 1949, Joyce Kligerman Newman, Two children

Lincensure: Illinois - 1946; New York - 1955; Colorado - 1960

Professional Societies: American College of Surgeons, American College of Chest Physicians, American Association for Thoracic Surgery, American Thoracic Association, American Society for Artificial Internal Organs, Society of University Surgeons, Societe Internationale de Chirurgie, Society of Thoracic Surgeons.

10035461 42

13. Publications

Giles F. Filley, M.D.

Wagner, W.W., Barker, D.B., and Filley, G.F.: A Photographic Method for Quantitating Blood Flow in the Pulmonary Microcirculation. J. Biological Photographic Assoc. 35: 95, 1967.

Filley, G.F.: Acid-Base and Blood Gas Regulation. Lea & Febiger, Philadelphia, March 1971.

Horsfield, D., Dart, G., Olson, D.E., Filley, G.F., and Cumming, G.: Models of the Human Bronchial Tree. J. Appl. Physiol. 31: 207, 1971.

Silvers, G.W., Maisel, J.C., Petty, T.L., Filley, G.F., and Mitchell, R.S.: Central Airway Resistance in Excised Emphysematous Lungs. Chest 61: 603, 1972.

Olson, D.E., Sudlow, M.F., Horsfield, K., and Filley, G.F.: Convective Patterns of Flow During Inspiration. Arch. Int. Med. 131: 51, 1973.

George W. Paul, Ph.D.

Paul, G.W., and Marc de Chazal, L.E.: Interfacial Tensions in Ternary Liquid-Liquid Systems. J. Chem. Engr. Data 12: 105, 1967.

Paul, G.W., and Marc de Chazal, L.E.: Correlation of Interfacial Free Energy in Binary and Ternary Systems. Ind. Engr. Chem. Fundamentals 8: 104, 1969.

Shoemaker, P.D., Paul, G.W., and Marc de Chazal, L.E.: Surface Tension of Simple Liquids from the Radial Distribution Function. J. Chem. Phys. 52: 491, 1970.

Paul, G.W., and Froning, H.R.: Salinity Effects in Micellar Flooding. J. Pet. Tech. 24: 957, 1973.

G. Wayne Silvers

Silvers, G.W., Maisel, J.C., Petty, T.L., Filley, G.F., and Mitchell, R.S.: Central Airway Resistance in Excised Emphysema Lungs. Chest 61: 603, 1972.

Maisel, J.C., Silvers, G.W., George, M.S., Dart, G.A., Petty, T.L., and Mitchell, R.S.: The Significance of Bronchial Atrophy. Am. J. Path. 67: 371, 1972.

Silvers, G.W., Maisel, J.C., Petty, T.L., and Mitchell, R.S.: Reduction in Peripheral Airway Resistance in Excised Emphysematous Lungs. Proc. 15th Annual Aspen Emphysema Conf. Chest 63: 32S, 1973.

Silvers, G.W., Maisel, J.C., Petty, T.L., Filley, G.F., and Mitchell, R.S.: Increase of Flow in Excised Emphysematous Lungs Following Lavage with Acetylcysteine or Salina. Am. Rev. Resp. Dis. 110: 170, 1974.

Silvers, G.W., Maisel, J.C., Petty, T.L., Filley, G.F., and Mitchell, R.S.: Flow Limitation During Forced Expiration in Excised Human Lungs. J. Appl. Physiol. 36: 737, 1974.

4

14. First year budget:

A. Salaries (give names or state "to be recruited")

Professional (give % time of investigator(s) even if no salary requested)	% time	Amount
Giles F. Filley, M.D. (Princip...l Investigator)	30%	—
George W. Paul, Ph.D. Postdoctoral Res. Fellow	100%	REDACTED
G. Wayne Silvers, Research Associate	50%	
Melvin M. Newman, M.D., Consultant	Not stipulated	—
Technical		
Marlyce George, Research Technologist	50%	—
Fringe Benefits		REDACTED
Sub-Total for A		

B. Consumable supplies (by major categories)

Chemicals & fixatives	750
Valves, tubing, glassware	350
X-ray room use fee & supplies	450
Recorder paper & developer	300
Suction filter	25
Sub-Total for B	1,875

C. Other expenses (itemize)

Publication costs	500
Maintenance and Modification of Equipment	1,000
Travel	500
Sub-Total for C	2,000
Running Total of A + B + C	35,573

D. Permanent equipment (itemize)

1 ea Model 1210 Harvard Peristaltic Pump	625
1 ea Model 550 Harvard Pump speed modulator	225
1 ea Large speciman plethysmograph	200
1 ea duNouy Tensiometer	495
1 ea Cathetometer telescope and stand	370
1 ea Gionometer Eyepiece	237
1 ea Statham transducer	350
1 ea Universal illuminator	111
Sub-Total for D	2,613
E. Indirect costs (15% of A+B+C)	5,336
Total request	43,522

15. Estimated future requirements:

	Salaries	Consumable Suppl.	Other Expenses	Permanent Equip.	Indirect Costs	Total
Year 2	R	1,500	2,000	—	5,664	43,427
Year 3		1,650	1,000	—	5,955	45,656

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5.

16. Other sources of financial support:
List financial support from all sources, including own institution, for this and related research projects.

CURRENTLY ACTIVE

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates
Cardiopulmonary Physiology	USPHS 3 T01 HL05450-14S1	36,423	4/1/74 - 6/30/75

PENDING OR PLANNED

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates
Human Pulmonary Surfactant Function in Situ	USPHS 1 R01 HL 17544-01A1	42,245	4/1/75 - 3/31/76
Surface Chemistry and Physics of Pulmonary Surfactant (Young Investigator) G.W. Paul	USPHS 1 R23 HL 18353-01	15,000	7/1/75 - 6/30/76

It is understood that the investigator and institutional officers in applying for a grant have read and accept the Council's "Statement of Policy Containing Conditions and Terms Under Which Project Grants Are Made."

Principal investigator

Typed Name Giles F. Filley, M.D.

Signature _____ Date Jan 21 '75

Telephone REDACTED
Area Code Number Extension

Checks payable to

iversity of Colorado Medical Center
Office of Grants & Contracts

Mailing address for checks

4200 East Ninth Avenue

Denver, Colorado 80220

Responsible officer of institution

Typed Name Harry P. Ward, M.D.

Title Acting Vice President for Health Affairs

Signature _____ Date 1-75

Telephone REDACTED
Area Code Number Extension

10035461 45

CTR Grant #

THE COUNCIL FOR TOBACCO RESEARCH - U.S.A., Inc.
110 EAST 59TH STREET
NEW YORK, N. Y. 10022

Project Title: Human Pulmonary Surfactant Function In Situ

We hereby certify that human subjects involved in this proposal to whom we administer investigational or any other procedures, including personality tests and questionnaires will have signed legal consent forms, and that human subjects below the age of 18 years will have legal consent forms signed by the parents, legal guardians, or probate court. If this is deemed unnecessary or undesirable in this particular instance, we state the reasons below.

1/21/75
Date

Signature of Principal Investigator or Program Director

1/22/75
Date

Business Officer of Appropriate Institution Authority
Harry P. Ward, M.D.
Acting Vice President for Health Affairs

Return ONE copy to: The Council for Tobacco Research-U.S.A., Inc.

G. F. Filley MD
#1021

VETERANS ADMINISTRATION
HOSPITAL
150 MUIR ROAD
MARTINEZ, CALIFORNIA 94553

IN REPLY
REFER TO:

David Stone, Ph.D.
Associate Research Director
Council For Tobacco Research - U.S.A., Inc.
110 East 59th Street
New York, New York 10022

Dear Doctor Stone:

I have read and evaluated the grant request of Giles F. Filley, M.D., "Human Pulmonary Surfactant Function In Situ". Let me say I am delighted with his innovative approaches, but I must weigh this against his naievete.

His proposal had three main parts. The first dealt with the distribution of the alveolar lining material in human lungs post-mortem. He is fully aware that preservation in situ of pulmonary surfactant has never been satisfactorily accomplished. Some observers have had partial success. One of the major problems is that the lipoprotein surfactant is readily soluble in glutaraldehyde as well as the dehydrating agent alcohol. Thus to use these agents to fix and dehydrate in preparation for electron microscopy, despite his innovative method, is open to question. He must prove that 1) No loss of surfactant occurrs in his fixation or dehydration process and more difficulty 2) No change in distribution during the same preparation. He has given no protocol on how he intends to insure these two features. Thus I find this portion unrealistic.

The second phase of his study seems more obtainable. The measurement of PV characteristics of small airways with and after removal of surfactant appears to hold genuine possibility of giving meaningful data. I am in favor of this portion of the protocol.

The third portion deals with the wettability and adhesiveness of pulmonary surfactant on dissected lung tissues. He proposes to suspend thin slices of alveolar tissues or small airways in a Wilhelmy film balance with the tissue substituting for the usual platinum float. He will attempt to estimate wettability by measuring the contact angle of surfactant solution and the suspended tissue. I find this portion of his proposal least acceptable. The cut surfaces

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Show veteran's full name, VA file number, and social security number on all correspondence.

of "alveolar tissue" would be next to impossible to suspend, to wash free of surfactant layers, to keep free from edema and trauma and denaturation. In addition the contact angle reflects the surface activity of the surfactant fluid as well as the wettability of the traumatized tissue. Sorting these factors out is worthy of Solomon.

In general, I was delighted by his innovative approaches. I feel that several phases are excellent and the others could be eliminated. I hope this will be helpful.

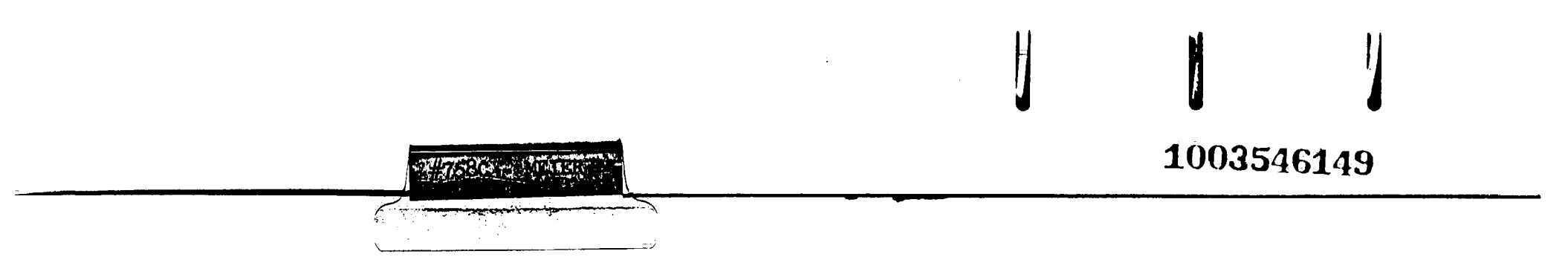
Sincerely,

Theodore N. Finley, M.D.
Professor of Medicine, U.C. Davis
Chief of Chest Medicine
V.A. Hospital, Martinez

TNF: cc
Enc:

Copies to:
Drs. Jacobsen, Liebow, Sommers, Gardner and Hochella.

31.9.75



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THE COUNCIL FOR TOBACCO RESEARCH–U.S.A., INC.

January 9, 1975

Grant Application No. 758C

CANCER

To: The committee comprising Drs. Feldman, Huebner, Jacobson

Subject: Hans Meier, D.V.M., The Jackson Laboratory, Bar Harbor, Me.
Continuation application No. 758C
"Oncogenesis in the rabbit: genetic susceptibility, vertical transmission of virus, and environmental influences."

History

Study supported by CTR since May 1, 1970. In 1972 SAB approved a three year study.

Request

Application No. 758C is for a continuance of studies currently underway, and requests $24,973 for the first year of a three year period.

Documents submitted (attached)

1. Application dated December 31, 1974 (23 pages) including C.V.'s of Drs. H. Meier and R. Fox.

2. Appendix (2 pages).

Comment

Dr. Meier also has submitted a first year renewal request (Grant No. 951R1) in the amount of $27,010 for the project entitled "Transplacental effects of nitrosocompounds in inbred strains of mice and rabbits".

D.S.

DS:wg
Encl.

=752C

THE COUNCIL FOR TOBACCO RESEARCH - U.S.A.

110 EAST 59TH STREET
NEW YORK, N. Y. 10022

RECEIVED
JAN 2 1975

Application For Research Grant

Date: 31 December 1974

1. Name of Investigator(s): (Include Title and Degrees)
HANS MEIER, D.V.M., Dr. med. vet., M.R.S.H., Senior Staff Scientist
RICHARD R. FOX, Ph.D., Staff Scientist

2. Institution & Address:
The Jackson Laboratory, Bar Harbor, Maine 04609

3. Short Title of Project:
Oncogenesis in the rabbit: genetic susceptibility, vertical transmission of virus, and environmental influences.

4. Proposed Starting Date:
1 July 1975

5. Anticipated Duration of this Specific Study:
Three (3) years

6. Brief Description of Objectives or Specific Aims:

Hereditary lymphosarcoma and immune hemolytic anemia associated with thymoma in rabbits provide important new models for study of the pathogenesis of neoplasia, including probable viral oncogenesis, and immunopathological disorders. A search for and propagation of oncogenic RNA virus(es) or genomes in rabbits is important because of (a) the widespread distribution of these viruses among vertebrates, (b) their possible role as universal determinants of cancer, (c) our preliminary evidence for the presence of C-type RNA virus and polymerase in these rabbits, and (d) the many experimental uses of rabbits in biomedical research.

We have three strains of rabbits that have pathologies relevant to this study of the interaction of host genotype, environment, and C-type RNA virus(es): strain WH with its hereditary lymphosarcoma (1); strain X with its hereditary autoimmune hemolytic anemia associated with thymoma (2), and strain III an ahh inducible rabbit strain highly susceptible to tumorigenesis induced by ethylnitrosourea (3) (see CTR #951 renewal application).

We are attempting virus-isolation following established procedures for murine, avian, and feline leukemia viruses. It should be possible to sediment virus from rabbit tissue by ultra- and gradient centrifugation. Isolated and purified virus can then be used as antigen(s) for the production of viral specific antisera, both against coat proteins and the group-specific antigen. We also need additional information to decide whether the same gene, which is responsible for susceptibility to hereditary immune hemolytic anemia, also predisposes to thymoma; and whether both hemolytic anemia and thymoma are due to an interaction with a vertically transmitted (inherited) C-type RNA genome. Because strain WH and X are genetically related, a common hereditary basis is being sought for all three conditions. Lines susceptible and resistant to tumorigenesis may be obtained within a strain.

7. Give a Brief Statement of your Working Hypothesis:

Because our studies of the lymphosarcoma and hemolytic anemia are compatible with the concept of both a genetic susceptibility and vertical transmission of a virus, we hypothesize that the two phenotypes are the result of a specific viral-host interaction.

10035461S1

8. Details of Experimental Design and Procedures: (Attach Separate Pages)

See attached.

9. Physical Facilities Available (Where Other than Administering Organization Indicate Geographical Location)

The Jackson Laboratory: Main Laboratory, Hamilton Laboratory, and Virus-Leukemia Laboratory.

10. Additional Requirements:

None.

11. Biographical sketches of all principal and professional personnel (append)

See attached.

12. List of publications: (Five most recent as pertinent) (append)

See attached.

13. Budget: (1st year)

	% time	Amount
A. Salaries (Personnel by names)		
Professional		
Hans Meier, Project Director	5	
R. R. Fox, Project Co-Director	15	
Technical		
D. D. Crary, Professional Assistant	10	
R. F. Norberg, Professional Assistant	20	
E. F. Farrin, Research Assistant	15	
Animal Caretaking		
Secretarial Assistance		
	Sub-Total	REDACTED
Employee benefits: 16% of Personnel		REDACTED
B. Consumable Supplies (list by categories)		
Chemicals, glassware, instruments		2,100
	Sub-Total	$ 2,100
C. Other Expenses (itemize)		
Domestic travel to scientific meetings		300
Publication costs		300
Art, Photo, and Replicating Service		200
Food and bedding		2,300
	Sub-Total	$ 3,100
D. Permanent Equipment (itemize)		
None		0
	Sub-Total	$ 0
E. Overhead (15% of A+B+C)		$ 3,257
	Total	$24,973

Estimated Future Requirements:

	Salaries	Consumable Suppl.	Other Expenses	Permanent Equip.	Overhead	Total
Year 2	REDACTED	$2,250	$3,350	—	$3,565	$27,333
Year 3	REDACTED	$2,550	$3,650	—	$3,928	$30,112

It is understood that the applicant and institutional officers in applying for a grant have read and found acceptable the Council's "Statement of Policy Containing Conditions and Terms Under Which Project Grants Are Made."

HANS MEIER
Signature
Director of Project (207) 288-3373, X 273
Telephone

AUSTIN C. CARTER
Signature
Business Officer of the Institution
(207) 288-3373, X 227
Telephone

100354615 3

Other Sources of Financial Support

List financial support for research from all sources, including own institution, for this and/or related research projects.

Current

Title of Project	Source	Amount	Duration
Oncogenesis in the rabbit: genetic susceptibility, vertical transmission of virus, and environmental influences. (#758)	The Council for Tobacco Research, U.S.A.	$ 24,401	7/1/74-6/31/75
Natural occurrence of RNA tumor virus (genomes) and host--gene control of their expressions.	National Cancer Institute	438,496	5/1/74-4/30/75
Hereditary diseases.	National Eye Institute	27,276*	6/1/74-5/31/75
Rabbit inbred and mutant stocks resource.	NIH Division of Research Resources	85,515	1/1/74-12/31/74
Transplacental effects of nitrosocompounds in inbred strains of mice and rabbits. (#951)	The Council for Tobacco Research, U.S.A.	25,461	7/1/74-6/30/75

Pending

Transplacental effects of nitrosocompounds in inbred strains of mice and rabbits.	The Council for Tobacco Research, U.S.A.	27,010	7/1/75-6/30/76
Hereditary diseases.	National Eye Institute	24,698	6/1/75-5/30/76
Rabbit inbred and mutant stocks resource.	NIH Division of Research Resources	40,000*	1/1/75-12/31/75

*Direct costs

BUDGET JUSTIFICATION

The studies proposed in this application take advantage of the availability of several strains of rabbits and mutant stocks at the Jackson Laboratory (see appendix). We expect them to prove of considerable value in studies of tobacco products.

Although a wide variety of spontaneous infectious and hereditary diseases have been found in the rabbit, tumors have been reported infrequently. However, only a few systematic studies have been conducted. Our studies reveal that lymphosarcomas and hemolytic anemia occur with high frequency in rabbits, but that the incidence, type, and development are greatly influenced by age, breed, and other constitutional factors. Clearly, studies with genetically controlled rabbits both supplement and complement studies with inbred mice. This unique resource of rabbits at the Jackson Laboratory must be maintained and made available to research workers elsewhere. The two strains of rabbits, WH and X, are extremely valuable for studies in oncogenesis but their exploitation has hardly begun. Strain III has an excellent research potential because of its aryl hydrocarbon hydroxylase (AHH)-inducibility. Fortunately, we have the professional staff and talent essential to the studies that we propose. The financial support requested from The Council for Tobacco Research for maintenance and study of these rabbits is minimal, but is adequate when coupled with existing support.

There is no need, at present, to include in this budget salary provision for 100% effort contributed by the project co-directors because this proposal relates to work supported by NIH research contract N01 CP 33255 from the National Cancer Institute and NIH resource grant RR 00251 from the Division of Research Resources of NIH.

8. DETAILS OF EXPERIMENTAL DESIGN AND PROCEDURE

Introduction and background

We stated in detail the aims of our studies in our applications (1970-1974) for Research Grant #758. Clearly, the work we proposed, particularly the viral studies, could not be completed within the period for which we received funding; at least 3 additional years of work and support are required. Thus, this application is for continuance of studies currently underway.

The overall goals of our studies remain basically as proposed previously:

1. We have observed within a few years over 93 cases of lymphosarcoma in a small breeding colony of strain WH rabbits, and affected animals of both sexes were found in each of several generations. Because of the unusual case aggregation of lymphosarcoma, we wish to investigate the host genetic factors conferring susceptibility to lymphosarcoma, the mode of inheritance or transmission, the probability of a vertically transmitted virus, and the environmental influences that may modify incidence and pathogenesis of lymphosarcoma.

2. Another strain of rabbits, strain X, which is genetically related to the WH strain, is characterized by a high incidence of immune hemolytic anemia (76 cases); and thymoma occurs as well. We want to find out the mode of inheritance or transmission of immune hemolytic anemia and thymoma in strain X rabbits; then, because the two strains are genetically related, we can evaluate the possibility of a common hereditary basis for all conditions in both strain X and WH. The various clinical or phenotypic expressions probably derive from differences in the genetic background of the two strains.

3. We believe that all three conditions are caused by a vertically transmitted virus analogous to the C-type RNA viruses occurring in a number of vertebrates, including man. The outcome of viral-host interaction depends to major degrees on host genetic factors, but it may be modified by environmental influences.

Specific aims

Studies of the interaction of host genotype, environment, and virus, if present, are complex and some narrowing of aims is necessary. Thus, the specific aims are:

1. To isolate and propagate a C-type RNA virus from rabbits,

2. To study its biological, biochemical, and biophysical properties, and

3. To relate its function, if any, to lymphosarcoma, hemolytic anemia, and thymoma.

Scientific progress during tenure of current grant

A brief background and resume of work accomplished thus far is pertinent to the studies proposed in this application. Detailed reports of our accomplishments have been submitted (see previous progress reports).

1. Assays and distribution of DNA-polymerases in rabbit tissues. RNA tumor virus(es) contain an enzyme, RNA-dependent DNA polymerase, that transcribes DNA from an RNA template. The role of this enzyme has not as yet been established,

i.e., whether or not it plays a part in transformation or may be essential for a transformed state.

Introduction and background

In collaboration with Dr. Masa Hatanaka and Dr. Gilden of Flow Laboratories, we assayed organs of normal, azathioprine (Imuran)-treated, and lymphosarcoma-afflicted rabbits. Most organs, both normal and malignant, revealed enzyme activity. Thymuses, major mesenteric and popliteal lymph nodes, and gastrointestinal tract have the highest specific activity of RNA-dependent DNA polymerase suggesting the common nature or origin of the lymphopoietic systems. Also, the data taken together with our serological findings (previously reported) verify the presence of an RNA viral genome in WH rabbits. It is also present in other incipient inbred strains of rabbits, i.e., III_{Dw} and a hybrid between two strains (III_{mo} x III_c) F_1.

1. We have observed [illegible]

2. Natural occurrence of RNA tumor viruses. Our earlier findings of interspecies group-specific antigen or gs-AG (gs-3) reactivity indicates that a type-C RNA viral genome must be present in the rabbit (4). Since complete C-particles are absent, the genome must largely occur in covert or incomplete form. However, there may be sites of predilection, e.g., bone marrow, progestational uterus, blastocyst, uterine secretions, etc., where complete infectious virus is expressed.

In the light of findings of C-type particles in early mouse embryos (5), we considered the possibility that the progestational and estrus uterus, blastocyst, and uterine secretions of various rabbit strains may harbor C-type particles. This approach coincides with that of Daniels (6) who accidently observed C-type particles in rabbit blastocysts in studies designed to evaluate the role of the uterus in "providing information" for the growth and differentiation of the embryo. Our preliminary findings were discussed in detail by Dr. Meier at the SAB meeting in Arizona in the spring of 1974.

3. Pedigree analyses for lymphosarcoma and immune hemolytic anemia susceptibilities. We have now observed 93 cases of lymphosarcoma in strain WH and seven cases in genetically related rabbits of strain AX. Autoimmune hemolytic anemia occurred in 76 rabbits of strain X; in addition, seven cases were found in strain AC which is in part derived from strain X. In fact, all affected individuals in the four strains are genetically related and trace back to a common ancestor, X974. Thus, we suspect that the two different syndromes, each caused by an autosomal recessive gene, ls and ha, respectively, may indeed be manifestations of the same gene with the phenotype dependent upon the remainder of the host genotype. To either confirm or rule out this possibility, we are now awaiting results of sufficient matings between ls/+ and ha/+ heterozygotes.

Certain of these aspects including our pathological and hematological findings in strains WH and X afflicted with lymphosarcoma and immune hemolytic anemia have been presented at the First Annual Veterinary Symposium of Hycel, Inc., (7) and in two book chapters (8, 9).

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4. Myeloid leukemia in strain III. We have reported the first case of myeloid leukemia in the rabbit. It occurred in a 13-1/2-month-old male of subline III_{ep}. Its features are distinct from hereditary lymphosarcoma by cell type, organ involvement, and distribution of tumors. Studies are in progress to determine whether this case of myeloid leukemia is of hereditary origin. Also, we plan to determine in additional cases whether an oncogenic type-C RNA viral genome is involved in this tumor, as well as in lymphosarcoma of WH rabbits (10).

5. Genetic predisposition to tumors in the rabbit. Our analysis of genetic

factors has been presented in Progress Report 2, and a paper describing our findings has been published (11).

6. Hereditary hemolytic anemia associated with thymoma in strain X rabbits. Autoimmune (Coombs-positive) hemolytic anemia occurs with high frequency in rabbits of strain X. This condition is rapidly fatal with a mean survival time of about 5 months. Sometimes it is associated with thymic hyperplasia and thymoma. The gene conferring susceptibility, designated ha, may be identical with that causing lymphosarcoma susceptibility and assigned the gene symbol, ls, in WH rabbits. Strains X and WH are closely related genetically, and a common gene responsible for all conditions may have phenotypic expressions that are dependent upon the remainder of the genotype. We are also considering the possibility that a vertically transmitted virus similar or analogous to the C-type RNA virus of mice is of etiological importance in addition to gene(s) conferring susceptibility (2).

Methods and procedures of proposed studies

In our studies of the pathogenesis of lymphosarcoma in WH rabbits and of hemolytic anemia associated with thymoma in strain X rabbits, we wish to achieve an understanding of the underlying mechanisms leading to each disorder in the two strains. No doubt the interactions of host genotype, "environment," and C-type RNA virus, if present, are complex. In the light of our findings to date we are proposing the following studies:

1. Characterization of viral protein markers. It appears that the C-type virus markers, group-specific antigen (gs-AG) and RNA-directed DNA polymerase, are present in rabbit tissues. We propose to study the chromatographic properties, template and cation preferences, and the sedimentation coefficient of the rabbit "viral" polymerase as well as its immunological relationship to known viral polymerases.

We found peaks of DNA polymerase activity, which correspond with MLV polymerase, when fractions from 10 to 30% glycerol gradients were surveyed with the template primer poly rA.oligo dT, whereas no peaks were detected with $(dT)_{12-18}$ alone (Fig. 1 and 2). The absence of activity in the presence of $(dT)_{12-18}$ rules out the possibility of contamination with terminal deoxyribonucleotidyl transferase. Polymerase activity was 10 times higher in the gestational uterine tissue than in the nongestational rabbit. A shift in peak positions occurred which may be due to the presence of stimulators and inhibitors from an impure preparation. Further purification by sephadex-chromatography, use of other template primers, and analysis of the immunological relationship between the rabbit polymerase and other known viral polymerases are necessary for the ultimate characterization of this enzyme.

Further support for the presence of C-type RNA virus and polymerase in rabbits is given by banding patterns (12) and electron microscopy (13). Figure 3 demonstrates that uterine fluid labelled with ^{3}H-uridine 24 hours before banding contains virus polymerase with a bouyant density of approximately 1.15 g/ml. Extensive electron microscopic studies of 5-day blastocysts and uterine fluid revealed the presence of budding type-C particles.

Extracts from the uterus and from other rabbit tissues (lymphosarcoma and normal tissues) will be passed over a microgranular DEAE-cellulose column (14). The viral reverse transcriptase, if present, and the cellular polymerase (DNA polymerase II) elute from the column at low salt concentrations and are thus separated from other cellular DNA polymerases (DNA polymerase I and III) which

Fig. 1. Reverse transcriptase activity from uterine tissue of 14-day pregnant strain III_{vo} rabbit.

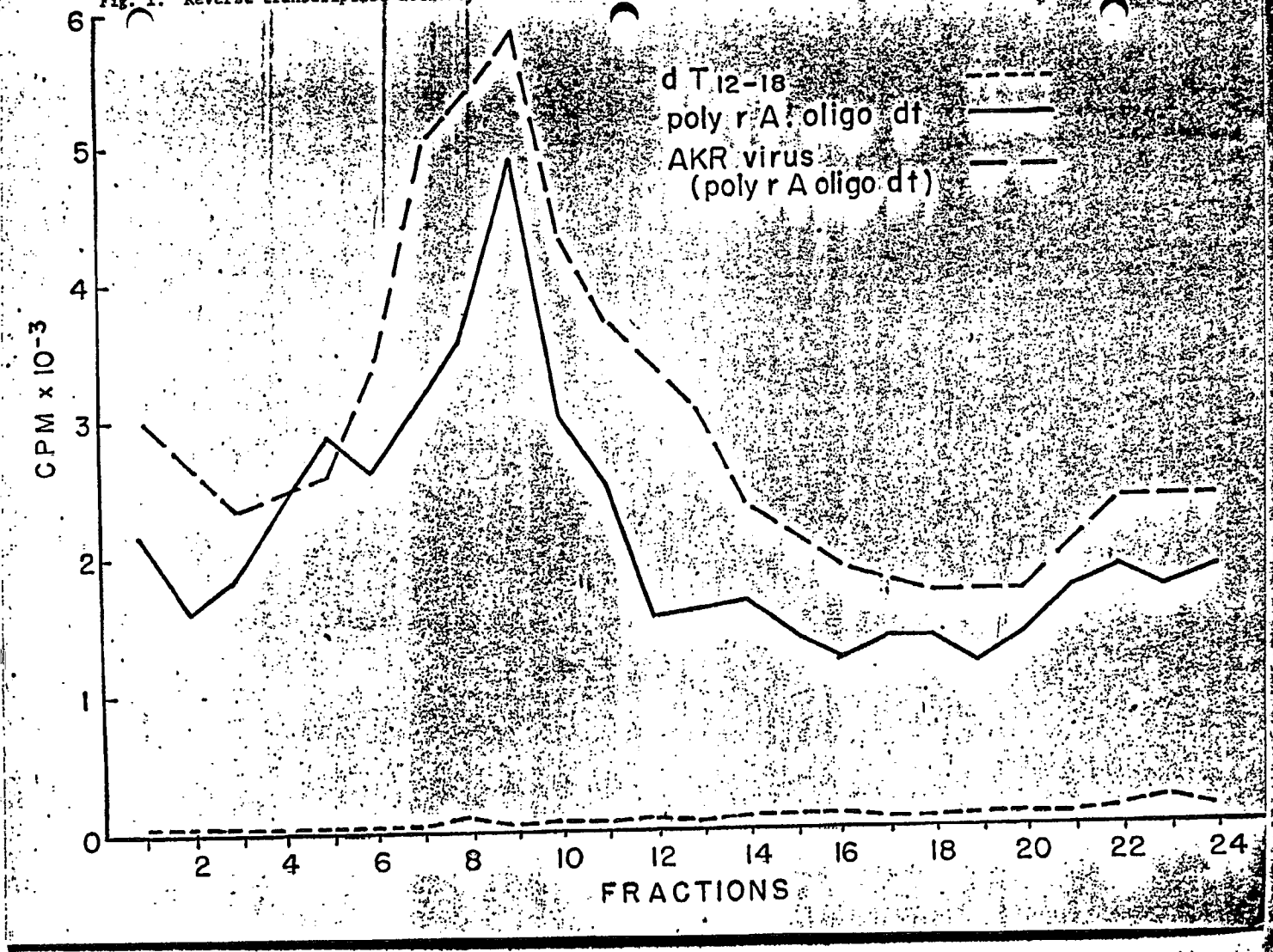


Fig. 2. Reverse transcriptase activity from uterine tissue of induced hydrouterus.

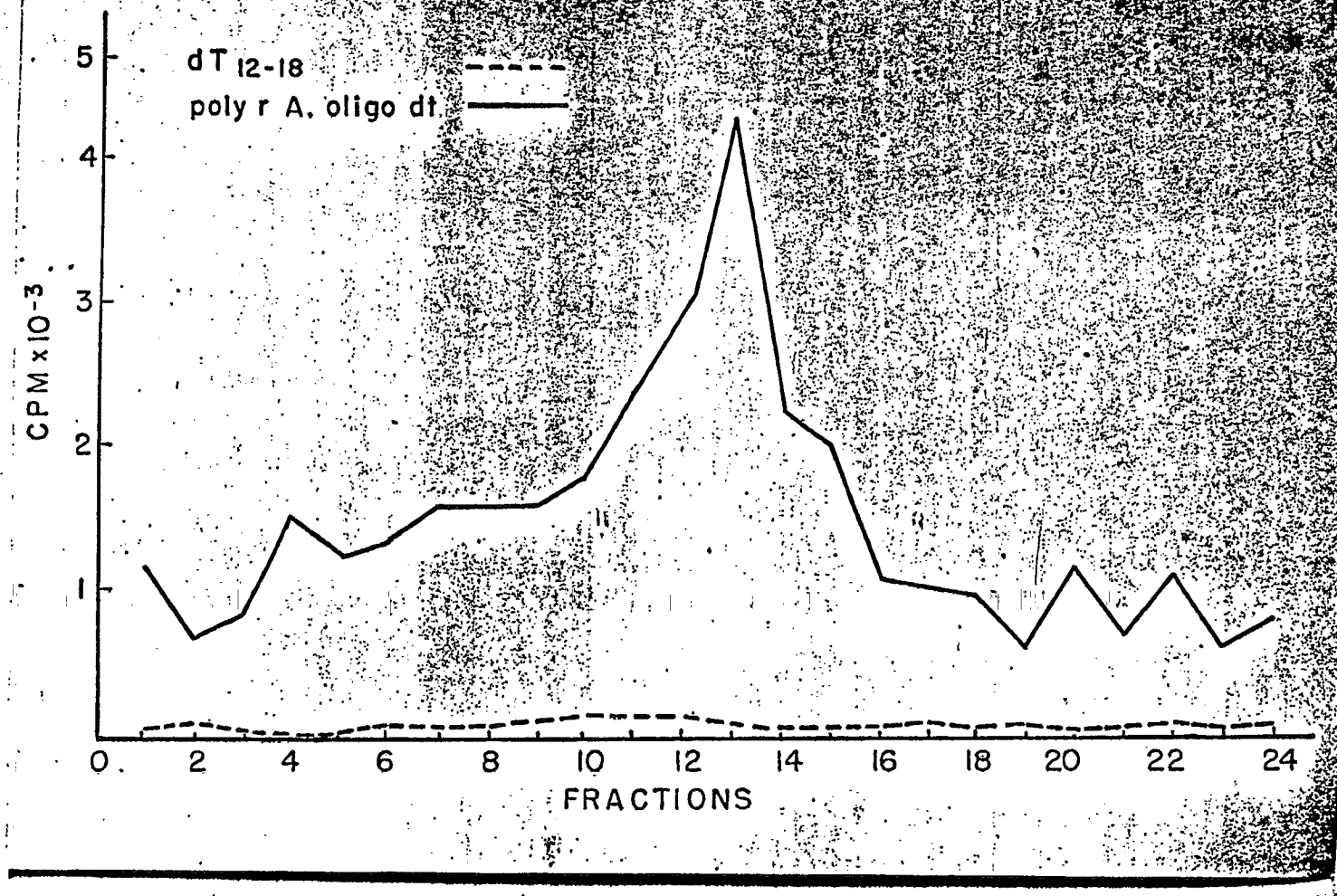
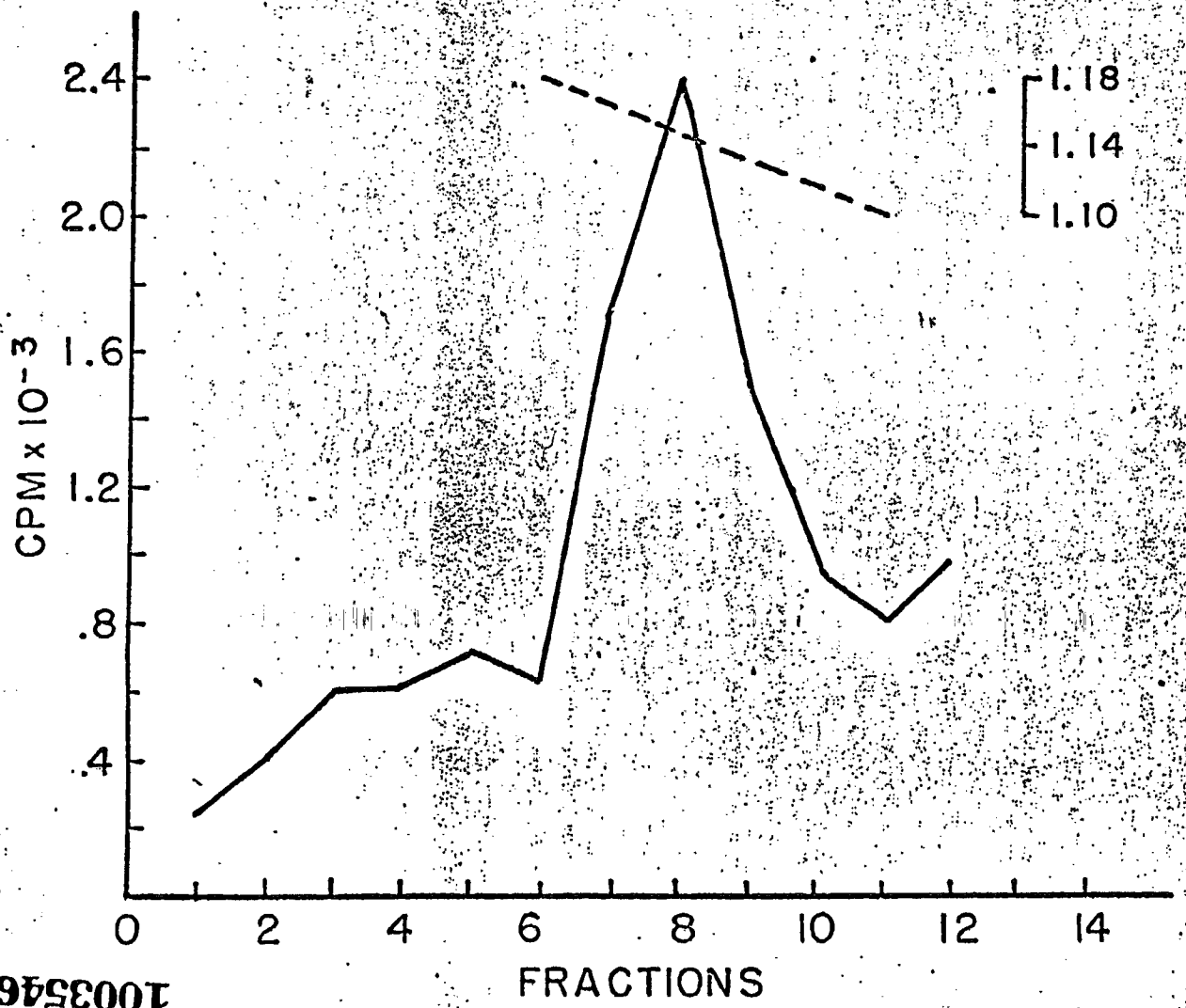


Fig. 3. Reverse transcriptase activity of uterine fluid from induced hydrouterus.



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elute at a higher ionic strength (14). The peak fractions, determined by absorbance at 260/280, from the phosphocellulose column eluting at 0.26 M KCl are then pooled and concentrated by dialysis against a polyethylene glycol buffer.

2. DNA polymerase assays. The DNA polymerase assays will be as described by Lewis (14) using various templates and concentrations of Mn^{2+} or Mg^{2+} as follows:

Template primer	Tritium-labeled substrate	Divalent cation
$(A)n.(dT)_{12-18}$	TTP	Mn^{2+} or Mg^{2+}
$(C)n.(dG)_{12-18}$	dGTP	Mn^{2+} or Mg^{2+}
$(dA)n.(dT)_{12-18}$	TTP	Mn^{2+} or Mg^{2+}
$(dT)_{12-18}$	TTP	Mn^{2+} or Mg^{2+}
$(dG)_{12-18}$	dGTP	Mn^{2+} or Mg^{2+}

3. Velocity gradient sedimentation. Samples of enzymes will be layered onto 5 to 20% sucrose gradients and centrifuged for 16 hours at 150,000 x g at 4°C in a Spinco 50.1 rotor. Fractions will be collected by bottom puncture and analyzed for DNA polymerase activity. Protein markers will be processed similarly on gradients and detected by absorbance at 280 nm (15).

4. Antibody inhibition studies. Antibody inhibition studies will be performed as described by Todaro (12). Antibody to the rabbit "viral" polymerase will be prepared in rats (16). A portion of the viral polymerase is incubated with an equal volume of antibody to known viral polymerase. A DNA polymerase assay is then performed to measure residual enzyme activity.

5. Attempts at isolation of rabbit C-type RNA virus. In the light of findings in inbred strains of mice, we shall consider the possibility that rabbits may harbor two types of viruses, ecotropic and xenotropic, as discussed below. Thus, our approaches to identifying these viruses include EM studies, cocultivations, RT assays (including simultaneous detection methods for both RT and 70S RNA), isopycnic zonal banding, mixed lymphocyte reactions (MLR in vitro), and graft-versus-host reaction (GvH) in vivo. Details of some of these approaches are described below; others have been described previously:

Our searches for ecotropic rabbit type-C RNA virus(es) will include tissue EM, RT assays, radioimmunoassay, hybridization reactions, and, isopycnic zonal banding of rabbit tissue culture (TC) supernates and uterine fluids.

a. Electron microscopic studies. We shall primarily focus on the pro-

gestational and estrus uterus, blastocysts, and pellets of uterine secretions. Virus particles have previously been found in mammalian embryos of several species and stages, particularly the mouse (13). Small A-type particles were common in early stages but absent in blastocysts, but C-type particles occurred only in blastocysts (5).

by Lewis (14) using various concentrations and concentrations of
foll Tissues are fixed in 3% glutaraldehyde in 0.1 M cacodylate buffer and then postfixed in 1% osmic acid. Postfixation is followed by dehydration in ethanol and embedded in Epon-Araldide (17). Ultra thin sections are cut on a Porter Blum I microtome and stained with uranyl acetate and lead citrate. Tissues will be examined for C-type particles in a Hitachi HU-11 C electron microscope.

b. Reverse transcriptase assays and isopycnic zonal banding. Uterine secretions and fluids of non-cocultivated rabbit cell cultures will be assayed for RT by a modified technique of Ross et al. (18). Uterine secretions and fluids from both progestational and estrus stages will be collected from rabbits following surgical cervix ligation. They will then be assayed for RT directly (18), pelleted, and pretreated for isopycnic banding (19). RT determinations are particularly useful for assay of low-titered viral preparations or with suitable viral infectivity assays lacking.

c. Simultaneous detection assay. The development of the "simultaneous detection assay" (SDA) directly provides evidence for the concurrent analysis of two unique characteristics of C-type RNA viruses, namely, a 70S viral RNA associated with an RNA-dependent DNA polymerase (20). In a reaction mediated by a C-type virus, ^{3}H-DNA will sediment in a 70S region of the gradient representing the 70S RNA:^{3}H-DNA reaction product. The successful use of the SDA to detect RNA viruses in mouse and human milk (20) may also be applicable for the detection of this reaction product in rabbits.

5 to Uterine fluid and tissues from rabbits with lymphosarcoma will be examined for the presence of 70S RNA:^{3}H-DNA as described by Schlom and Spiegelman (20). The sensitivity of the assay makes it a useful tool for detecting the presence of C-type virus in rabbit tissues.

Biophysical properties can be utilized to indicate the presence of viral agents in culture (21). The RNA tumor viruses band at a density of 1.16 to 1.18 g/ml in a continuous sucrose gradient (15% to 60%).

Rabbit cell cultures are injected with labelled uridine and the fluid is collected 24 hours later; alternatively, growth medium containing 20 uCi/ml of ^{3}H-uridine are added to subconfluent cultures and incubated for 24 hours at 37°C. Supernates or fluids are then examined as described by Panen and Kirstein (21), and Kruse and Patterson (22).

d. Purification of group-specific (gs) antigen. High speed virus pellets from culture fluids are disrupted and gs-antigen (P30) purified by phosphocellulose chromatography and pressure dialysis using the same procedures for the purification of RT and described by Ross and Scolnick (18) and Scolnick et al. (23).

e. Radioimmunoassay of gs-antigen. Interspecies (gs-3) antigen is determined by a competitive radioimmunoassay (16) using ^{125}I Rauscher murine leukemia virus gs-antigen purified by gel filtration and isoelectric focusing (23). Protein is determined by the method of Lowry et al. (24).

f. Extraction of cellular DNA and RNA. Rabbit cells are suspended in three volumes of 0.05 M Tris-HCl, pH 8.3, 5 mM magnesium acetate, and 0.04 M sodium chloride, homogenized, and centrifuged at 10,000 x g. The pellet is resuspended in 20 volumes of the buffer adjusted to 1% sodium dodecyl sulfate, and extracted at room temperature with chloroform-isoamyl alcohol (24:1 V/V) and with neutralized water-saturated phenol containing 10% m-cresol. After phenol extraction, the solutions are extracted four times with ether to remove the phenol and treated with 0.5 N KOH at 49°C for 12 to 16 hours to hydrolyze RNA. The remaining DNA is neutralized and dialyzed against three changes of 300 volumes of 0.01 M Tris-HCl, pH 7.4, 0.1 M NaCl, 10^{-4} M EDTA, and stored at -20°C at a concentration of approximately 8 mg/ml. One gram (wet weight) of cells should yield about 2 to 5 mg of DNA. Analysis of the optical density profile of this DNA on Cs_2SO_4 gradients are not expected to reveal RNA in the preparations. Cellular RNA is extracted as previously described by Benveniste et al. (25).

g. Synthesis and purification of viral ^{3}H-DNA. The endogenous reverse transcriptase reaction from detergent-disrupted rabbit type-C virus is used to synthesis (^{3}H)thymidine-labeled DNA in the presence of actinomycin D (50 ug/ml) as described previously by Benveniste and Scolnick (26). The specific activity of the ^{3}H-DNA is 2.0×10^7 CPM/ug.

h. Hybridization reactions. Approximately 2000 counts/min (0.1 ng) of enzymatically synthesized DNA is incubated with either cytoplasmic RNA or with DNA in 10^4- to 10^7-fold excess for 48 to 72 hours at 31°C in 0.20 ml reaction mixtures containing 0.015 M Tris-HCl, pH 7.4; 0.15 M sodium chloride; 5×10^{-4} M EDTA; 0.1% SDS, and 38% formamide. The extent of hybrid formation can be detected by hydrolysis with purified S_1 nuclease as described previously by Benveniste and Scolnick (26) and Benveniste et al. (25).

Our searches for xenotropic rabbit type-C RNA virus(es) will consist of MLR, graft-versus-host reaction (GvH), RT, cocultivations, and focus assays.

a. Mixed lymphocyte culture reactions (MLR) and graft-versus-host (GvH). The evidence that viral oncogenesis can be enhanced by immunosuppression is now overwhelming (27). A combined electron microscope and virologic analysis by Schwartz et al. (28) showed that MLV could be activated during the GvH and MLC reaction in mice. A similar procedure will be followed in rabbits using (III x WH)F_1 with one of the parents as the donor of lymphocytes. In the GvH each F_1 will receive four intraperitoneal injections of 60×10^7 cells once a week for 4 weeks. The F_1 rabbits will be killed 10 days after the last injection. Spleens will be taken for EM, culturing, and polymerase assay. Control rabbits will include: (1) Normal F_1 rabbits with no treatment; (2) F_1 rabbits injected with adjuvant; (3) F_1 rabbits given sheep red blood cells; (4) F_1 rabbits given allogenic spleen cells; and (5) F_1 rabbits given parental spleen cells which have been treated in vitro with mitomycin C before injection. Each control group will receive treatment once a week for 4 weeks.

The counterpart in vitro of the GvH reaction, the MLC reaction, will be examined for the induction of C-type virus in the rabbit. Lymphocyte suspensions will be prepared from spleens of young rabbits [strain III, WH, and (WH x III)F_1]. Spleens will be minced, passed through progressively smaller syringes, and put through a Ficoll-gradient as described by Kruse and Patterson (22) (bone marrow). For the MLC reaction, 2.5×10^6 cells per ml of either strain III or WH will be incubated with 2.5×10^6 cells per ml F_1 lymphocytes in sealed test tubes in volumes of 3 ml for 6 days. The cultures will be supplemented with 1 ml of RPMI 1640 at 48-hour intervals and assessed for cell proliferation by the addition of 1 uCi

of ^{3}H-thymidine to 1 ml of the cultures 4 hours before the termination of the experiments in a liquid scintillation counter. Cell proliferation is assessed by precipitation with trichloracetic acid. [illegible] at room temperature with chloroform-isoamyl alcohol (24:1 v/v) and with ethanol. Control cultures include (a) strain III or WH and F_1 lymphocytes incubated alone and (b) F_1 lymphocytes incubated with mitomycin treated strain III or WH lymphocytes. Supernatants from all cultures will be assayed for virus production by the reverse transcriptase reaction. [illegible] pH 7.4, 0.1 M NaCl, 10^{-3} M EDTA, and stored at -20° C at a concentration of approximately [illegible]. In inbred strains of mice, GvH occurs across the major histocompatibility (H-2) region (29). However, analysis of a large number of H-2 crossovers and their parental strains revealed that the strongest GvH reaction was associated with the Ir (immune response)-region (29). Apparently, the products of these genes are receptors on the surface of thymus-derived lymphocytes (T-cells). Histocompatibility loci exist as well in the rabbit; the major locus, RL-A, is similar to the Ag-B of rats and H-2 of mice (30). Thus, it is likely that Ir genes are present also, and we may take advantage of Ir differences in attempts to activate endogenous type-C virus. Both eco- and X-tropic viruses have been activated in mice by MLV and GvH.

b. Cocultivation and focus formation. Tissues for cell cultures will be removed asceptically from various organs of normal and lymphosarcomatous rabbits of different strains, WH, X, III, etc; cultures will be established according to standard procedures (22) and subcultured after confluency using 0.1% trypsin in Hank's balanced salt solution.

The rabbit-cell cultures as well as those cells used for cocultivation are maintained in plastic (Falcon) flasks containing Eagle's minimum essential medium (EMEM) supplemented with 10% fetal calf serum, 0.3% glutamine, and gentomycin (0.6 ml/100 ml medium). Cells used for cocultivation are non-virus-yielding newborn rat kidney cells transformed by the Harvey strain of murine sarcoma virus (NRK-Harvey or H-NRK), human rhabdomyosarcoma (RD), and human embryo skin-muscle fibroblasts (HESM), NRK, and various BALB/c virus (MuLV)-negative cell lines (A31, R4, and S16). Their use and derivation has been amply documented (31-34). The cocultivation procedures are those described by Levy (35). Briefly, we shall follow these lines:

Direct cocultivation of 4×10^5 rabbit cells with 1×10^4 Harvey virus transformed newborn rat kidney cells (H-NRK); the culture fluid will be changed every 2 to 3 days and the 7-day fluid collected.

An alternate procedure consists of cocultivating rabbit cells with rhabdomyosarcoma cells (RD), human embryonic skin and muscle cells (HESM), and BALB/c cells separately, following pretreatment with DEAE-dextran (25 ug/ml). After 7 days the respective cultures will be trypsinized and split. One dish will be cocultivated with the H-NRK, whereas we shall grow the second dish for another week before cocultivation.

The 7-day cocultivation fluids will then be assayed for RT and focus formation on normal rat (NRK), human (RD, HESM), mouse (BALB/c-A31, R4, and S16), and rabbit embryo cultures as well as for gs-antigen and hybridization reactions as previously described. Xenotropic viruses would be expected to grow on sensitive foreign host (heterologous) cells but not homologous cells (35).

6. Studies in strain X rabbits. Information is needed to decide whether (a) the same gene that is responsible for susceptibility to hereditary immune hemolytic anemia also predisposes to thymoma, (b) the gene giving rise to hemolytic anemia or thymoma, or both, in strain X is the same gene that is responsible for lymphosarcoma-susceptibility in WH rabbits, and (c) both hemolytic anemia and thymoma are due to an interaction with a vertically transmitted C-type RNA viral genome.

The following procedures should provide the answers sought to the first two questions. Information on the third will result from approaches identical or analogous to those described for WH rabbits.

a. Is the same gene responsible for both hemolytic anemia and thymomagenesis? We are chronically treating strain X rabbits homozygous for the hemolytic anemia trait (ha/ha) with the immunosuppressive drug, azathioprine (Imuran). Azathioprine should prevent its occurrence because the hemolytic disease is due to an immune disorder. However, by analogy to NZB mice, it should not interfere with development of thymoma. Thus, in order to establish that the gene is responsible for both conditions, we must be able to selectively induce thymoma upon continued azathioprine therapy.

b. Are the genes, lymphosarcoma-susceptibility (ls) and hemolytic anemia (ha), identical? In order to answer the question of allelism of ha and ls, F_1 hybrids between respective heterozygotes (ls/+ and ha/+) should yield approximately 25% abnormal offspring if the two genes are allelic. We cannot of course decide *a priori* whether they resemble either of the respective homozygotes (ls/ls and ha/ha) or whether they have clinical features of both.

Genetic studies are time-consuming and require large numbers of animals. Also, we cannot know *a priori* what the latency period may be for clinical signs to appear if heterozygotes (ls/ha) are obtained.

In past and current breeding experiments we have made the following crosses, ha/ha x ls/+ and ha/+ x ls/+; indeed, several presumptive heterozygotes (ls/ha) have been observed. These findings clearly indicate the identity of ha and ls. In order to obtain a representative spectrum of phenotypic expression of compound heterozygotes, we anticipate a requirement of at least 10 afflicted rabbits.

So far, affected rabbits suffered either from hemolytic anemia or a combination of hemolytic anemia and a lymphoproliferative disorder depending upon the age at which clinical signs appeared. Although we now have nine afflicted progeny (compound heterozygotes), a number of potentially affected rabbits are still alive.

7. Both strains of rabbits. Strains WH and X are partially inbred. The coefficients of inbreeding, which defines that proportion of loci for which the original or base population was heterozygous but which through inbreeding has become homozygous, are approximately 0.72 and 0.88, respectively. We estimate, therefore, that each strain may be homozygous for as many as 80% of the initially variable loci and that these loci have been fixed for different alleles in the two strains because of deliberate selection for and maintenance of specific mutant genes.

A population of animals need not be inbred for an analysis of the inheritance of a specific gene or phenotypic trait. The genetic basis of a trait by which two strains differ can be obtained by crosses to obtain F_1, F_2, and backcross generations. From a population segregating in Mendelian proportions for recognizable phenotypes, one may estimate the number of gene pairs that distinguish the two parental strains with respect to the trait in question. Conversely, because there is reduced genetic variability within inbred strains, they are unsuitable for selection experiments unless genetically heterogeneous populations are synthesized by crossing two or more strains. Because the WH and X strains are now only partially inbred, we can select for those genes that produce a particular phenotype of interest, e.g., lymphosarcoma susceptibility and resistance. Thus, in each strain we should be able to produce two or more lines that may vary in tumor development and also produce a line that is tumor-resistant. These may interact specifically, but variously in studies of chemical or other cocarcinogenesis.

In addition to the 93 cases of lymphosarcoma in strain WH, we have now also found seven cases in genetically related rabbits of strain AX. Also, in addition to the 76 cases of autoimmune hemolytic anemia in strain X, seven cases were observed in strain AC, which is in part derived from strain X. In fact, all affected individuals in all four strains are genetically related and trace back to a common ancestor, X974.

We are now looking for circulating antinuclear and anti-DNA antibodies in the various rabbit strains, as well as immune complex disease in biopsies from kidneys. Because lymphoid cell lines maintained in suspension on a gyratory shaker have yielded complete virus from NZB and related mice, we are using analogous conditions for cell lines produced from spleens and lymph nodes.

Significance of this research

The proposed studies relate to an opportunity for the analysis of two major groups of disorders: cancer and autoimmune disease. They do not deal directly with the effects of tobacco, but are clearly relevant to tobacco effects in several indirect ways: (a) as neither tobacco nor its various chemically defined components nor all known carcinogenic chemicals provide a host with the genetic information to produce or induce cancer and any other disease, (b) the occurrence of cancer or any non-neoplastic disease is dependent upon the inborn host-genetic regulation of all processes allowing or disallowing it to occur. Thus, an analysis of the hereditary pathways and their acquired modifications through tobacco or other means is fundamental to an understanding of all disease processes. Some of these may be attached by use and study of the two mutants (ls/ls and ha/ha) of rabbits. Because of their analogies, they may help clarify mechanisms of the respective human disorders and provide basic information about their pathogenesis.

So far, we have made three major findings: (a) the likely identity of the genes conferring susceptibility to both cancer (lymphosarcoma) and immune hemolytic anemia (autoimmune disease), (b) the most probable presence in WH, X, and other rabbit strains of an endogenous oncogenic C-type RNA tumor viral genome, and (c) strains WH

and X have Coombs' autoantibodies. The major significance of our studies lies in the potential relationship of these three observations.

A population of animals need not be inbred for an analysis of the inheritance of a 1. Overall significance. Like human systemic lupus erythematosus (SLE) and the related disease in New Zealand Black (NZB) mice and (NZB x NZW)F_1 hybrid mice, the hereditary autoimmune disease in strain X rabbits is associated with synthesis of various autoantibodies to erythrocytes and various nuclear materials (36). Some of the possibilities for their occurrence are that: (a) The hosts are unable to repair nucleic acids properly, and altered nucleic acids might either have an abnormal catabolic fate or antigenicity. This situation would be analogous to the defect in the repair of DNA that occurs in xeroderma pigmentosum. (b) The hosts are deficient in or have abnormal nucleases, and perhaps other catabolic enzymes, that result in the formation of immunogenic breakdown products from spent, autologous cells. (c) The hosts have a defect in some intracellular structure such as the nuclear or plasma membrane resulting in an inability to keep nucleic acids in a proper configuration or an intracellular compartment. (d) The hosts harbor a virus whose genome is immunogenic because of a different configuration or nucleotide composition from that of the host, thereby terminating tolerance to autologous nucleic acids (Meier, unpublished).

Thus, it seems possible that cells from patients with SLE or from diseased NZB mice are variants of one or more of the defects listed. Their detection and evaluation is clearly important in elucidating the pathogenesis of SLE, as well as the catabolism, structure, and repair of nucleic acids in normal cells.

In addition to autoantibodies to double-helical DNA, DNA-histone complexes, single-stranded DNA, and nucleolar RNA and their complexes, NZB mice also make antibodies to the endogenous C-type RNA tumor virus (36). The presence of such a genome in both strains WH and X, as well as all other rabbit strains is most probable. Thus, with genes ls and ha being identical and conferring susceptibility to either lymphosarcoma or autoimmune hemolytic anemia, a common pathogenesis of the two disorders is likely.

We now have evidence for a highly significant association in mice between the endogenous C-type RNA viral genome and tumorigenesis (37). In fact, viral expression in early life is a highly predictable marker for tumorigenesis with advancing age. This expression is host-gene controlled, and relates to tumors of all types, i.e., mesenchymal as well as epithelial tumors, and leukemias as well as solid tumors (37). Thus, the mechanism for tumorigenesis is hereditary or "built-in," but whether or not tumors will develop depends upon other host-genes as well as environmental factors (37). Although this explanation requires ultimate substantiation, it provides the most rational basis for all available tumor data.

2. Specific significance of project. Rabbits are of considerable value in biomedical research because of the vast amount of morphologic, physiologic, genetic, and biochemical data available, the simplicity of their care and breeding, and their large size. The finding of lymphosarcoma in the rabbit and its hereditary basis provides a new and important model for studies of the pathogenesis of neoplasia (1). The rabbit colonies at the Jackson Laboratory are free of Shope papilloma and fibroma, and myxomatosis viruses. Except for a small number of epithelial tumors, which have been described (11, 38), most tumors in our rabbits have been of lymphoid tissue origin, i.e., lymphosarcoma and thymoma.

Affected WH rabbits usually die between the age of 5 and 13 months. The

neoplastic involvement of lymphoreticular and other organs, especially kidneys, corresponded to a pattern observed in lymphosarcoma of other domestic animals (4). Specifically, it resembled in many ways visceral lymphosarcomatosis of cats that has been proved unequivocally to be caused by feline leukemia virus (1, 4). Similarities between rabbit and cat lymphosarcomas include the sites of onset, distribution of the neoplastic lesions, and the finding of a predominantly aleukemic hemogram (1, 4). However, in rabbit lymphosarcoma, we often found a relative increase in lymphoid cells including both immature and atypical forms (1).

If a C-type RNA virus is demonstrated, the *1s* and *ha* genes may confer susceptibility to malignant transformation of lymphoreticular tissues. We have found a number of genes in mice that enhance oncogenesis, especially leukemogenesis, but that do not influence the presence or absence of either murine leukemia virus (MuLV) or MuLV antigens (39).

If complete virus is inducible or is spontaneously expressed in any strain of rabbits, its isolation and purification is essential for the production of specific antisera. Also, yet another species may be demonstrated to harbor type-C RNA tumor viruses.

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	Member, Scientific Advisory Board, The Council for Tobacco Research, USA	1971 -
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Major interests:	Experimental pathology, cancer	

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1954 - 1957	Angell Memorial Hospital, Boston, Fellow, Resident, Chief of Pathology
1955 - 1957	Harvard Medical School, Course Work in pathology and neuro-pathology
1957	Harvard University, Summer School of Arts and Sciences, and of Education (Biophysics and radioactive measurements)
1958	Armed Forces Institute of Pathology (Course in histochemistry)
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1965-	The Jackson Laboratory, Staff Scientist
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12. RELEVANT PUBLICATIONS

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APPENDIX 1: CHARACTERISTICS OF RABBIT STRAINS AT THE JACKSON LABORATORY AS OF 15 OCTOBER 1974.

STRAIN (Name & Symbol)	COLOR	PREDOMINANT GENOTYPE	ORIGIN	COEFFICIENT OF INBREEDING[1]	PREDOMINANT ALLOTYPE	MATURE WEIGHT (GM)	OTHER GENES SEGREGATING
III (sublines)	Albino	cc AA E^dE^d as as	New Zealand White Castle '32	0.98	$a^1a^1b^4b^4c^7c^7$	3800	l, scoliosis[2]
(III)				(0.98)			
(III vo)		(rere)		(0.97)			
(III ep)		(ep/ep)		(0.96)			
(III ah)		(Ah/ah)		(0.96)			
(III cd)		(Cd/cd)		(0.97)			
(III Da)		(Da/da)		(0.55)			
(III Dw)		(Dw/dw)		(0.95)			
(III vo)		(Vp/vp)		(0.95)			
III_c	Albino	cc AA E^dE^d mpmp	Subline of III	0.94	$a^3a^3b^4b^4c^7c^7$	4000	
X	Sooty yellow	C(c^{ch2}) aa ea bb Haha AsAs	Castle's small race	0.88	$a^3a^3b^4b^4c^7c^7$	2200	dk, c21, as
WH	Agouti	AA EE WHWH Lsls[5]	Rockefeller Inst. 1949. Crossed with strains X, III, etc.	0.72		2300	e, r^2, E^d
AC[3]	Black, recessive white marking	aa E^dE^d, du^du^d Acac[5], Sbsb[5]	Dutch	0.85	$a^3a^3b^5b^5c^7c^7$	2400	y, du^w, c^{21}
ACEP[3]	Blue eyed white	vv, epep	Dutch	0.92	$a^3a^3b^4b^4c^{21}c^{21}$	2400	hg,
ACCR (B)[3][6]	Albino	cc sasa	Dutch	Inbred	$a^3a^3b^4b^4c^7c^7$	2400	y
(Y) (6)	Albino	cc sasa	Dutch	Inbred	$a^3a^3b^4b^4c^{21}c^{21}$	2400	c^7
OS	Black, minimal recessive white marking	aa E^dE^d, du^du^d, Osos[5]	Dutch Rockefeller Institute 1948	0.85	$a^2a^2b^4b^4c^7c^7$	3200	hydrocephaly[2]
AX	Chinchilla	$c^{ch3}c^{ch3}$ AA vv Axax[5]	Outcross of Chinchilla race V to strains III and X	0.68	$a^3a^3b^4b^4$	3500	du, bu, c^{ch2} c, hypospadias
AX_{bubu}	Albino	cc bubu	AX strain	0.76	$b^4b^4c^7c^7$	3500	c^{ch3}, c^{ch2}, a^1a
A	Albino	cc	Miscellaneous	0.95	$a^3a^3b^4b^4$	2700	l
C	agouti or black	AA or aa	Miscellaneous	0.98		2800	b, c, du

(APPENDIX 4 CONT.)

Genes of importance to studies of constitutional disease being maintained in the colony are:

Achondroplasia (ac)
dach (Da) (6)
dwarf (Dw) (4)
renal cysts (rc)
mandibular prognathism (mp)
hypogonadia (hg)
lymphosarcoma (ls)
hemolytic anemia (ha)

angora (1)
furless (f)
rex^2 (r^2)
satin (sa)
adrenal hyperplasia (ah)
chondrodystrophy (cd)

ataxia (ax)
buphthalmia (bu)
epilepsy (audiogenic seizures, ep)
gamma globulin alleles, Aa^1, Aa^2, Aa^3
gamma globulin alleles, Ab^4, Ab^5
gamma globulin alleles, Ac^7, Ac^7-
osteopetrosis (os)
spina bifida (sb)

Lethal muscle contracture, hypognathia, splay leg, diminutive dwarf, and cleft palate occur sporadically in some strains and may be genetic.

For other gene symbols, see:

Sawin, P. B. 1955. Recent genetics of the domestic rabbit. Adv. Genet. 7:183-226.
Robinson, R. 1958. Genetic studies of the rabbit. Bibliog. Genet. 17:229-558.
Fox, R. R. 1974. Taxonomy and Genetics In Biology of The Laboratory Rabbit, p.1-22. S. W. Weisbroth, A. L. Kraus, R. E. Flatt (eds) Academic Press

Footnotes:

(1) Maximum coefficient in the strain computed according to Wright's formula for coefficient of inbreeding (F). Inbreeding is by sib mating or as close to sib mating as possible consonant with maintenance of the specific lethal or semi-lethal genes (indicated by underlining) and an optimal reproductive capacity and viability.

(2) Level of penetrance is dependant upon environmental conditions.

(3) Sublines of the same Dutch stock obtained from Rockefeller Institute in 1948.

(4) Formerly symbolized dw, then recognized in the heterozygote (Sawin 1955 Adv. Genet. 7:183) and the symbol changed to Dw to reflect a semidominant.

(5) The ac, Da, os, sb, ha, ls, ah, cd, and ax genes are maintained in their respective strains by progeny testing of prospective parents. Homozygous transmitters are obtainable from the same test.

(6) The ACCR (B) and ACCR (Y) strains are also referred to simply as strain B or Y respectively.

(7) Formerly da, now recognized, by ear papilla, in the heterozygote.